b.) Remarks

Claims 21 and 22 have been amended in order to recite the present invention with the specificity required by statute. The subject matter of the amendment is explicitly disclosed in the specification as filed, *inter alia*, at page 9, lines 12-22. Accordingly, no new matter has been added.

Claims 21, 22 and 24-33 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly and distinctly claim the subject matter regarded as the invention. The basis for this rejection lies in the Examiner's contention that treated product of cells necessarily includes separated cell components (despite the fact that Applicants are clearly entitled to be their own lexicographer and have made abundantly plain on the record what such praseology means). Accordingly, in response claim 21 is amended to specify that the treated products are cells (and claim 22 is amended to specify that the first treated product is cells). This rejection is mooted.

Claims 30 and 33 are rejected for lack of biologic deposit. As understood, there are still two points remaining concerning this rejection: the availability of plasmid pAMoERSAW1 and of Namalwa KJM-1 cells. Regarding the latter, the Examiner states the cells are available only from Mr. F. Klein. This is not so, these cells were deposited by Mr. Klein and are available from the ATCC for $235 (CRL-1432).[1] Regarding the plasmid, the Examiner notes that the references discussed previously are not of record and such preparation "is not always simple". In response, copies of those references are enclosed and are identified on the enclosed Form PTO-1449 (attached at Tab A) together with JBC, discussed at footnote 1. As to whether or not initial production of the plasmid

[1] Such is also taught in *J. Biol. Chem.*, Vol. 276, Issue 5 (Feb. 2, 2001) 3498:507.

was simple or not for the inventors who prepared it (c.f., page 8, line 8 of the Office Action) such inquiry is off-point; even if the initial preparation was obvious, the procedure is now well-characterized, even if a considerable amount of work is involved. *Ex parte Forman*, 230 USPQ 546 (PTO Bd. Pat. App. Intf. 1986).

Claims 21, 22 and 24-33 are also rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not adequately described in the specification for the reasons set forth at pages 9-17 of the office action. This rejection, frankly, is without basis in law or fact since the terms objected to are explicitly disclosed in the specification as filed. Nonetheless, solely in order to reduce the issues, this rejection is overcome by the amendment to claims 21 and 22 specifying that the relevant treated products are cells.

As to dependent claims 30-33, the animal cell line may be any recombinant cell line, so long as it is capable of expressing a glycosyltransferase. Accordingly, cells having special function are not required. Additionally, as to the concern that β1,3-galactosyltransferase gene is not characterized in the present specification, β1,3-galactosyltransferases (and methods for obtaining them) are generally known at the time the present application was made. See *Ovid: Bibliographic Records*, attached at Tab A to the September 20, 2004 Preliminary Amendment. The specification does not teach, and preferably omits, what is well-known in the art. *Spectra-Physics Inc. v. Coherent, Inc.* 827 F.2d 1524 (Fed. Cir. 1987).

Claims 21, 24 and 25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Caputto or Herscovics, either in view of the common knowledge in the

art. Claims 22, 34, 25, 27 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Tochikura in view of Ichikawa, in further view of common knowledge. In support of the rejection, the Examiner points out (i) the claims do not recite extracellular UDP-glucose production, and (ii) uridine transport was expected.

This rejection is respectfully traversed in view of the foregoing amendment and the following remarks regarding the prior art.

I. Caputto

Orotic acid, uracil, orotidine and uridine are compounds which become UDP-glucose precursors on the metabolic pathways. However, they are not metabolized only for the biosynthesis of UDP-glucose, but are involved in myriad other pathways and are metabolized to a multiplicity of disparate compounds (see the copy of "http://hpv-web.lanl.gov/stdgen/bacteria/t_pal/images/tp-pyrimidine.html" attached at Tab B). This was common knowledge at the time the present application was filed. Also, Caputto neither teaches nor suggests that a sugar nucleotide can be efficiently produced by yeast using orotic acid, *etc.* Moreover, such production of the sugar nucleotide was not common knowledge at the time the present invention was made. If the Examiner disagrees, Applicants respectfully request that they be provided with a reference showing the same or an affidavit of the Examiner's personal knowledge in conformity with MPEP §2144.03.

II. Herscovics

Herscovics suggests but does not demonstrate that *S. cerevisiae* may biosynthesize UDP-GlcNAc intracellularly based on the sugar chain structure. Also, as described above, production of a sugar nucleotide using orotic acid, *etc.* was not common knowledge prior to Applicants' invention.

III. Tochikura

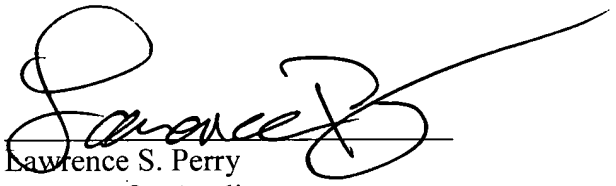
Tochikura produce UDP-glucose only using UMP as a precursor, and does not disclose or suggest producing UDP-glucose using orotic acid, uracil, orotidine or uridine as a precursor. As shown in the attachment at Tab B, orotic acid, uracil, orotidine and uridine are not metabolized only to UDP and it was not known, at the time the present application was filed, whether any of orotic acid, uracil, orotidine and uridine were effective for efficient production of UDP-glucose.

In view of the above amendments and remarks, Applicants submit that all of the Examiner's concerns are now overcome and the claims are now in allowable condition. Accordingly, reconsideration and allowance of this application is earnestly solicited.

Claims 21, 22 and 24-33 remain presented for continued prosecution.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,



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FORM PTO 1449 (*modified*)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

LIST OF REFERENCES CITED BY APPLICANT(S)
(*Use several sheets if necessary*)

ATTY DOCKET NO.	APPLICATION NO.
00766.000019.1	10/781,010

APPLICANT: Satoshi Koizumi, et al.

FILING DATE	GROUP
February 18, 2004	1652

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES/NO/ OR ABSTRACT

OTHER DOCUMENT(S) (*Including Author, Title, Date, Pertinent Pages, Etc.*)

		Cytotechnology, Vol. 3 (1990), p. 133
		Journal of Biochemistry, Vol. 101(1987), p. 1307
		Gene, Vol. 33 (1985), p. 103
		Proc. Natl. Acad. Sci. USA, Vol. 78 (1981), p. 1527
		Virology, Vol. 141 (1985), p. 30
		Nature, Vol. 313 (1985), p. 812
		J. Biol. Chem., Vol. 276, No. 5 (2001), p. 3498-3507

EXAMINER	DATE CONSIDERED

**EXAMINER:* *Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.*

J. Biochem. 101, 1307–1310 (1987)

A New SV40-Based Vector Developed for cDNA Expression in Animal Cells

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A useful vector, pAGE103, has been developed for the expression of cDNA in animal cells using the simian virus 40 (SV40) expression signals. cDNA could be expressed easily by inserting it into the multiple cloning sites (*Hin*dIII, *Sal*I/*Acc*I, *Xba*I, *Bam*HI, *Sma*I/*Xma*I, *Kpn*I/*Asp*718, *Sac*I and *Eco*RI) of the vector, which are located between the SV40 early promoter and the SV40 early RNA processing signals for splicing and polyadenylation. In addition to the above transcription unit, pAGE103 contains the replication origin of ColE1, and a dual Km^R/$G418^R$ selective gene. Several unique restriction sites are located on the boundaries between the above-mentioned three components of the vector, allowing the easy substitution or insertion of other genetic elements. The human interferon-β gene was inserted into pAGE103 and shown to be expressed transiently in COS-1 cells and stably in several animal cell lines.

In order to elucidate the functions of eukaryotic genetic elements or the gene products themselves, it is desirable to transfer a cloned gene back into animal cells and examine its functions. For the efficient expression of cDNA in animal cells, a promoter and RNA processing signals for splicing and polyadenylation have to be attached to the cDNA to reconstitute a transcription unit. Vectors containing such expression signals have been developed for the expression of cDNA and bacterial genes (*1*). Among plasmid vectors including the SV40 expression signals, the pKCR (*2*), pSV2-X (*3*), and pcD-X (*4*) vectors have been widely used for the expression of many genes. In these vectors, a gene is inserted into a specific restriction site(s) so as to be expressed under the control of the SV40 early promoter. Recombinant plasmids containing the replication origin of SV40 can replicate in COS cells which constitutively synthesize the SV40 large-T antigen (*5*), and enhance the transient expression of the inserted gene. For permanent expression, a second transcription unit of a selective marker gene should be inserted into the plasmid, or the plasmid should be cotransformed with another plasmid carrying a selective marker gene.

In this report, we describe a new SV40-based expression vector, pAGE103, developed for the expression of cDNA in animal cells. pAGE103 has the following advantages over the above-

Abbreviations: SV40, simian virus 40; Km, kanamycin; MCS, multiple cloning sites; kb, kilobase pairs; bp, base pairs; HuIFN-β, human interferon-β; HSVtk, *Herpes simplex* virus thymidine kinase; DME, Dulbecco's modified Eagle's medium.

mentioned vectors: i) it contains multiple cloning sites (MCS) between the SV40 early promoter and the SV40 early RNA processing signals for splicing and polyadenylation, and cDNA could be expressed easily by inserting it into an appropriate restriction site(s) in MCS; ii) it also contains a hybrid Tn5-derived Km^R/$G418^R$ transcription unit as a dual dominantly selectable marker which can be expressed in both *Escherichia coli* and animal cells (*6*). Thus, pAGE103 carrying cDNA can confer G418 resistance to animal cells; iii) it also contains four unique restriction sites (*Xho*I, *Eco*RV, *Cla*I, and *Pvu*I), which are located on the boundaries between the components of the vector, allowing the easy substitution or insertion of other genetic elements; and iv) even though it is compact and has a small molecular size, 4.1 kb, there is sufficient containment of genetic elements. The usefulness of pAGE103 was proved by its successful application to the expression of the human

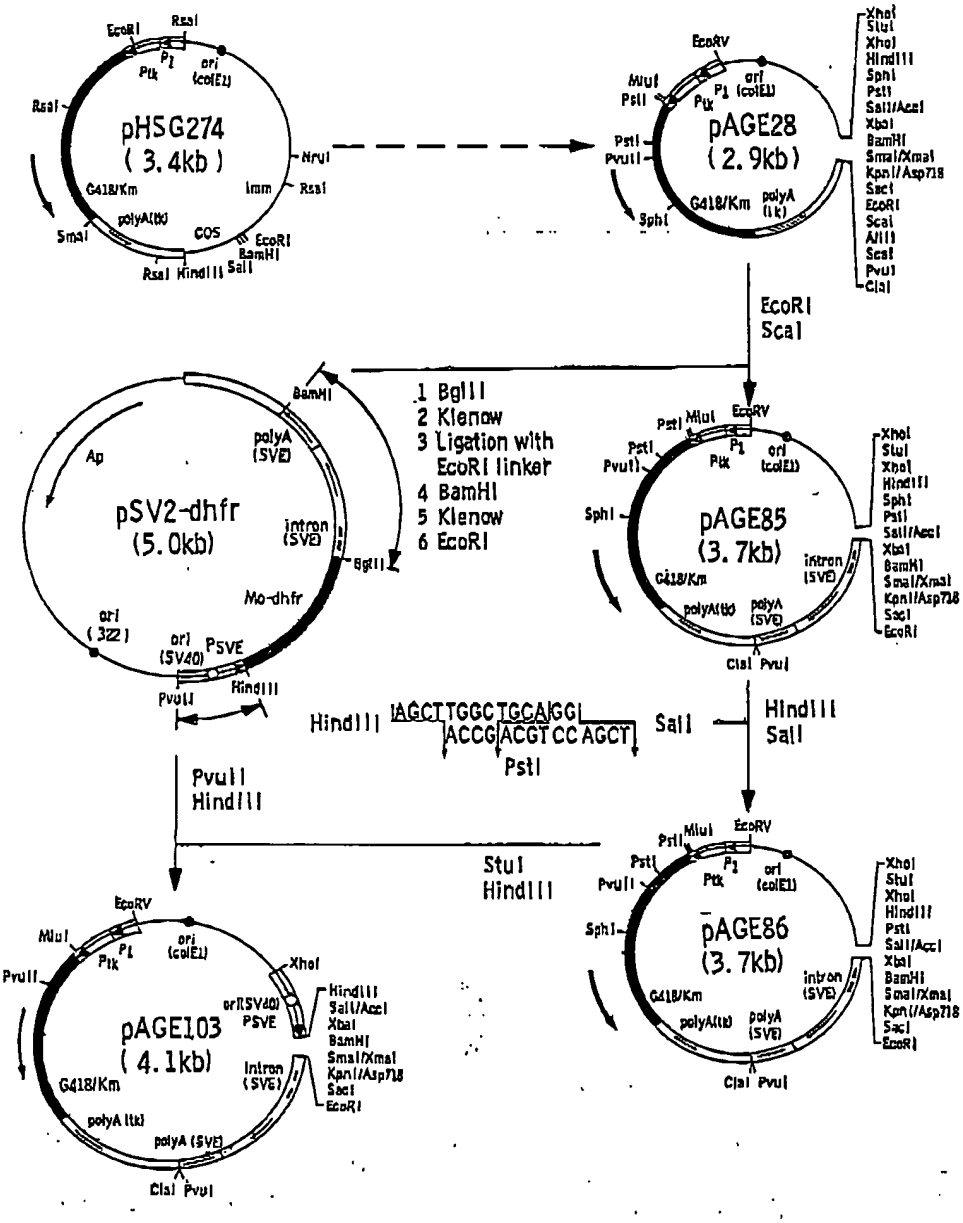

Fig. 1. Constru
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interferon-β (HuIFN-β) gene.

The construction of pAGE103 is explained in detail in Fig. 1. As a first step to obtain pAGE103, pAGE28 was constructed from pHSG274 (*6*), a cosmid vector containing a hybrid Tn5-derived $Km^R/G418^R$ gene. pAGE85 was constructed by inserting the SV40 early splicing and polyadenylation signals derived from a pSV2-X vector, pSV2-dhfr, into pAGE28 between the *Eco*RI-*Sca*I sites in MCS. Before inserting the SV40 early promoter into pAGE85, a *Sph*I site in MCS was removed by substituting a 14-bp *Hin*dIII-*Sal*I fragment derived from MCS of pUC9 for sequences between the *Hin*dIII and *Sal*I sites of pAGE85, because an ATG sequence in its recognition sequence (GCATGC) might be recognized as an initiation ATG codon instead of an original initiation ATG codon derived from an inserted gene according to the "scanning model" proposed by Kozak (*9*). pAGE103 was constructed by inserting the SV40 early promoter derived from pSV2-dhfr into pAGE86 between the *Stu*I-*Hin*dIII sites in MCS.

The utility of pAGE103 for expression of the HuIFN-β gene was examined. The HuIFN-β structural gene was excised from pHIFβ-121–312 (*11*), which contains the HuIFN-β chromosomal gene, by digesting it with *Hin*cII. The resulting 0.77-kb fragment containing a part of the 5′-noncoding region, the HuIFN-β coding region without an intron (*11*), and the whole 3′-noncoding region was inserted into the *Sma*I site of pAGE103 to yield a HuIFN-β expression plasmid, pSE1β1-2.

First, the transient expression of the inserted HuIFN-β gene in pSE1β1-2 was examined. As pAGE103 contains the replication origin of SV40 and does not contain the so-called "poison sequences" in pBR322 that inhibit plasmid replication in mammalian cells (*6*), pSE1β1-2 can replicate efficiently in COS cells and therefore enhance the expression of the inserted HuIFN-β gene. COS-1 cells and CV-1 cells, which are untransformed parental cells of COS-1, were transfected with pSE1β1-2, and then the levels of HuIFN-β were assayed (Table IA). The COS-1 cells transfected with pSE1β1-2 produced a high level of HuIFN-β, while the CV-1 cells similarly transfected produced an undetectable level of HuIFN-β.

Secondly, the permanent expression of the inserted HuIFN-β gene in pSE1β1-2 was examined in stably transformed cell lines. Various established cell lines of human, monkey, hamster, rat, and mouse origin were transfected with pSE1β1-2, and $10–10^2$ G418-resistant colonies were obtained through transfection with 10 μg of the plasmid DNA. The levels of HuIFN-β produced by the transformants from each cell line are shown in Table IB. The levels differed with the host cell line used, the transformant of a hamster cell line, BHK-21, exhibiting the highest level. It remains to be elucidated what factor(s) caused the

Fig. 1. Construction of a cDNA expression vector, pAGE103. pAGE28 was constructed from pHSG274 as follows: i) A 0.87-kb *Hin*dIII-*Nru*I fragment containing the λphage *cos* region and the ColE1 immunity region was removed from pHSG274, and then MCS partly derived from those of pUC19 (*7*) were inserted instead. ii) An *Rsa*I site, which is located on the boundary between the replication origin of ColE1 and the $Km^R/G418^R$ gene, was converted to a unique *Eco*RV site. iii) An *Eco*RI site, which is located in the promoter region of the *Herpes simplex* virus thymidine kinase (HSVtk) gene, and a *Sma*I site, which is located between the Tn5 $Km^R/G418^R$ gene and the polyadenylation site of the HSVtk gene, were removed to make the *Eco*RI and *Sma*I sites in MCS unique. pAGE85 was constructed from pAGE28 as follows. First, pSV2-dhfr (*8*) was digested with *Bgl*II, and then its cohesive end was filled in with *E. coli* DNA polymerase I Klenow fragment (Klenow fragment). After ligation with an *Eco*RI linker, the DNA was digested with *Bam*HI, and its cohesive end was filled in with Klenow fragment, and then the DNA was digested with *Eco*RI. The resulting 0.85-kb fragment containing the SV40 early splicing and polyadenylation signals was purified and then ligated with *Eco*RI and *Sca*I-digested pAGE28 to yield pAGE85. pAGE86 was constructed by inserting a 14-bp *Hin*dIII-*Sal*I fragment derived from MCS of pUC9 (*10*) between the *Hin*dIII and *Sal*I sites of pAGE85. For the construction of pAGE103, pSV2-dhfr was digested with *Pvu*II and *Hin*dIII, and the resulting 0.34-kb fragment containing the SV40 early promoter was purified and inserted between the *Stu*I and *Hin*dIII sites of pAGE86 to give pAGE103. Only restriction sites which cut pAGE103 uniquely are shown in the map for pAGE103. The following abbreviations are used: G418/Km, the Tn5-derived $G418^R/Km^R$ gene; Ap, the pBR322 ampicillin resistance gene; Mo-dhfr, the cDNA for mouse dihydrofolate reductase; P1, the pBR322 P1 promoter; Ptk, the HSVtk promoter; P_{SVE}, the SV40 early promoter; intron(SVE), the intron from the SV40 small-t antigen gene; polyA(SVE), the polyadenylation signal from the SV40 early gene; polyA(tk), the polyadenylation signal from the HSVtk gene; ori(colE1), the replication origin of colE1; ori(322), the replication origin of pBR322; ori(SV40), the replication origin of SV40.

TABLE I. Production levels of HuIFN-β in different host cells.

A. Transient expression[a]

Cell type	HuIFN-β activity (units/10^6 cells·24 h)
COS-1	1,000
CV-1	<4

B. Permanent expression[b]

Cell type	HuIFN-β activity (units/10^6 cells·24 h)
HeLa	436
CV-1	1,300
BHK-21	9,110
CHO-K1	1,290
Rat2	1,060
L-M (TK$^-$)	1,330

[a] 3×10^5 COS-1 and CV-1 monkey cells were transfected with 5 μg of pSE1β1-2 in 0.7 ml of Dulbecco's modified Eagle's medium (DME) containing 500 μg/ml of DEAE-dextran (*12*). After transfection for 8 h, the cells were washed twice with serum-free DME, fed with DME supplemented with 10% fetal calf serum and then incubated for 72 h. Antiviral activity of the HuIFN-β in the supernatants was measured by means of a cytopathic effect inhibition assay (*13*) using FL cells challenged with vesicular stomatitis virus. One unit of HuIFN-β was calculated by calibration against a National Institute of Health (NIH) reference standard for HuIFN-β, catalog number G-023-902-527. [b] 5×10^5 HeLa (human), CV-1 (monkey), BHK-21 (hamster), CHO-K1 (hamster), Rat2 (rat), and L-M(TK$^-$) (mouse) cells were transfected, respectively, with 10 μg of pSE1β1-2 by the calcium phosphate precipitation technique. $10-10^3$ G418^R transformants were obtained for each transfection. They were pooled and grown into mass cultures. The antiviral activity of HuIFN-β in the supernatants was measured as described above.

high expression of the transfected HuIFN-β gene in BHK-21.

Recently, a cloning vector called pDSP1, which has similar properties to pAGE103, was reported (*14*). pDSP1 contains two transcription units: the first unit expresses an *E. coli gal*K gene using the SV40 early gene expression signals which are bordered by MCS, and the second unit expresses an *E. coli xgprt* gene as a selective marker under the control of the SV40 early promoter and enhancer. pAGE103 has the following advantages over pDSP1. i) The HSVtk promoter, which is located upstream from the G418^R gene, does not contain an enhancer, and therefore the SV40 enhancer is the only enhancer located on pAGE103. By substituting different promoters and enhancers for the SV40 early promoter and enhancer in front of the inserted gene, the relative expression in different host cells can be closely examined. ii) The insertion of cDNA into pAGE103 is made easier by the existence of a greater number of unique restriction sites in MCS and by that there is no necessity to replace the other gene. iii) The structure of pAGE103 (4.1 kb) is more compact than that of pDSP1 (7.0 kb), and it contains a hybrid Km^R/G418^R transcription unit as a dual selective marker.

We wish to thank Miss M. Odaka for her excellent technical assistance, and Drs. T. Taniguchi, T. Hashimoto-Gotoh, and M. Oishi and his colleagues for the generous gifts of material. We are also grateful to Dr. M. Oishi for the useful discussions.

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Elsevier

GENE 1167

Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors

(Recombinant DNA; molecular cloning; polycloning sites; progressive deletions)

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SUMMARY

Three kinds of improvements have been introduced into the M13-based cloning systems. (1) New *Escherichia coli* host strains have been constructed for the *E. coli* bacteriophage M13 and the high-copy-number pUC-plasmid cloning vectors. Mutations introduced into these strains improve cloning of unmodified DNA and of repetitive sequences. A new suppressorless strain facilitates the cloning of selected recombinants. (2) The complete nucleotide sequences of the M13mp- and pUC-vectors have been compiled from a number of sources, including the sequencing of selected segments. The M13mp18 sequence is revised to include the G-to-T substitution in its gene II at position 6 125 bp (in M13) or 6967 bp in M13mp18. (3) M13 clones suitable for sequencing have been obtained by a new method of generating unidirectional progressive deletions from the polycloning site using exonucleases III and VII.

INTRODUCTION

Single-stranded DNA isolation has been facilitated by the properties of the single-stranded bacteriophage M13 (Messing et al., 1977). Though it is not a naturally transducing system, recombinant DNA techniques have been used to construct a general transducing system where double-stranded DNA can be introduced into the double-stranded RF of the phage. Upon transfection of appropriate host cells, the DNA strand ligated to the (+) strand of the RF is strand-separated, packaged and secreted without cell lysis as a recombinant single-stranded DNA phage.

Although inserts seven times longer than the wild-type viral genome have been cloned in M13 (Messing, 1981), the presence of large inserts can cause deletions. Accelerated growth of phage containing smaller inserts that arise from deletions makes maintaining large-fragment clones difficult (Messing, 1983). Differential growth can be observed in the plaque-size variety that results from infection of M13 clones possessing inserts of > 2000 bp.

With the introduction of a universal primer (Heidecker et al., 1980), M13 was used primarily for

Abbreviations: Ac, activator; Ap, ampicillin; B-broth, Bacto-tryptone broth; Cm, chloramphenicol; *Δ*, deletion; DTT, dithiothreitol; EMS, ethylmethane sulfonate; Exo III and VII, exonuclease III and VII; HA, hydroxylamine hydrochloride; IPTG, isopropyl-*β*-D-thiogalactopyranoside; LB, Luria broth; M13UC, see RESULTS, section c2; moi, multiplicity of infection; pfu, plaque-forming units; PHS, primer hybridization site; ^{R}, resistance; RF, replicative form; RT, room temperature; Sm, streptomycin; STE, 10 mM NaCl, 10 mM Tris·HCL pH 7.5, 1 mM EDTA; Tc, tetracycline; Xgal, 5-bromo-4-chloro-indolyl-*β*-D-galactopyranoside; YT, yeast tryptone; [], indicates plasmid-carrier state; *Δ*, deletion.

the subcloning or shotgun cloning of small fragments (for review, see Messing, 1983). Cloning of 10 000 bp fragments now appears possible with deletions occurring less frequently than previously predicted. One of the factors interfering with the cloning of large fragments is the restriction of unmodified DNA. JM103, a restrictionless host strain, was developed to circumvent this problem (Messing et al., 1981), but later lost the mutation (Felton, 1983; Baldwin, T., personal communication, C.Y.-P. and J.M., unpubl.), and instead carried a P1 lysogen which also contains a restriction and modification system.

This paper describes the construction and characterization of a number of new strains developed for use with the M13 and pUC cloning vectors. Each strain carries a specific set of mutations that help prevent various cloning problems. A complete listing of M13mp18 and pUC19 sequences and restriction sites is also presented. The M13mp sequence was compiled from published data (Van Wezenbeek et al., 1980; Messing et al., 1977; 1981; Farabaugh, 1978; Dickson et al., 1975; Kalnins et al., 1983; Messing and Vieira, 1982; Norrander et al., 1983). Re-sequencing of pUC was necessary, as a number of mutations had been introduced into the pBR322 region of the pUC vectors. A new method of generating unidirectional deletions from a full-length M13 clone of pUC6 by Exo III and VII was used for this purpose. The results have been combined with those published earlier (Ruther, 1980; Vieira and Messing, 1982; Rubin and Spradling, 1983; Stragier, P., personal communication).

TABLE I

E. coli strains and genotypes

E. coli strain	Genotype
JM83	*ara, Δ(lac-proAB), rpsL(=strA), φ80, lacZΔM15*
JM101	*supE, thi, Δ(lac-proAB),* [F', *traD36, proAB, lacI^{q}ZΔM15*]
JM105	*thi, rpsL, endA, sbcB15, hspR4, Δ(lac-proAB),* [F', *traD36, proAB, lacI^{q}ZΔM15*]
JM106	*endA1, gyrA96, thi, hsdR17, supE44, relA1, λ$^-$, Δ(lac-proAB)*
JM107	*endA1, gyrA96, thi, hsdR17, supE44, relA1, λ$^-$, Δ(lac-proAB),* [F', *traD36, proAB, lacI^{q}ZΔM15*]
JM108	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)*
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ$^-$, Δ(lac-proAB),* [F', *traD36, proAB, lacI^{q}ZΔM15*]
JM110	*rpsL, thr, leu, thi, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ(lac-proAB),* [F', *traD36, proAB, lacI^{q}ZΔM15*]
DH1	F$^-$, *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ$^-$*
GM48	*thr, leu, thi, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44*
SL10	Hfr H, *thi, sup^0, Δ(lac-proAB), galE, Δ(pgl-bio)*
TD1	MC4100, *recA56, srlC300::Tn10*
MC4100	*araD, rpsL, thi, Δ(lacIPOZYA)U169*
SK1592	*thi, supE, endA, sbcB15, hsdR4*
71-18	*Δ(lac-proAB), thi, supE,* [F', *proAB, lacI^{q}ZΔM15*]

MATERIALS AND METHODS

(a) Strains

The bacterial strains listed in Table I are *E. coli* K-12 derivatives. SK1592 was obtained from Sidney Kushner, DH1 from Jurgen Brosius, GM48 and HB101 from Raymond Rodriguez, and SL10 and TD1 from James Fuchs. Phages *λb2c* were from Bruno Gronenborn, and f2 from David Pratt. Strains were tested for relevant markers by standard methods and as described below.

(b) Maintenance of strains

Long-term storage of desired strains was accomplished by mixing 1 ml of a stationary-phase culture with 1 ml of glycerol and freezing at −70°C. Bacteria were revived by streaking aliquots on appropriate selective media and incubating at 37°C. Short-term working strain stocks were maintained at −20°C. Bacteria were revived by inoculating 10 ml of broth with 0.05 ml of the stock and shaking overnight at 37°C. Alternatively, strains containing the F′ from JM101 were maintained on glucose minimal plates for 2–4 weeks at 4°C.

(c) Media

Bacterial strains were grown in 2YT or LB broth (Miller, 1972) supplemented with 15 μg/ml Tc, 500 μg Sm/ml, or 50 μ
teria were plated on M
mal medium plus 1.5
supplemented with th
ml): 1 μg thiamine, 4
5-fluorocytosine, 40 μ
and 500 μg Sm. Fusa
plus 1.5% agar; Malo
medium (Miller, 1972
yeast extract and 1.4%
1972), or MacConkey
plates were supplemen
0.004% Xgal per plat
stored as 2% and 0.1

(d) Reagents

Tc, Sm, Cm, Ap, a
nalidixic acid, chlorot
obtained from Sigma
yeast extract, and Ba
from Difco. Boehring
Xgal. Restriction en
by Amersham, Beth
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(e) Transductions

Lysates of P1Cm1, c
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(f) Matings

Matings using F′ a
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(g) Curing of transpos

Elimination of the
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and Nunn, 1981).

(h) Transformations

Preparation of com
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ublished data (Van
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al., 1975; Kalnins
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; Rubin and Sprad-
l communication).

Table I are *E. coli*
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l plates for 2–4

, in 2YT or LB
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500 μg Sm/ml, or 50 μg/ml Ap when required. Bacteria were plated on M-9 minimal plates (M-9 minimal medium plus 1.5% agar; Miller, 1972) and supplemented with the following as required (per ml): 1 μg thiamine, 40 μg nalidixic acid, 20.1 μg 5-fluorocytosine, 40 μg amino acids, 12.5 μg Cm, and 500 μg Sm. Fusaric acid medium (Tcs broth plus 1.5% agar; Maloy and Nunn, 1981), B-broth medium (Miller, 1972) supplemented with 0.3% yeast extract and 1.4% agar, 1XA medium (Miller, 1972), or MacConkey plates were also used. B-broth plates were supplemented with 0.1 mM IPTG and 0.004% Xgal per plate. The Xgal and IPTG were stored as 2% and 0.1 M stocks, respectively.

(d) Reagents

Tc, Sm, Cm, Ap, amino acids, 5-fluorocytosine, nalidixic acid, chlorotetracycline, and IPTG were obtained from Sigma. Agar, MacConkey agar, yeast extract, and Bacto tryptone were obtained from Difco. Boehringer Mannheim supplied the Xgal. Restriction endonucleases were provided by Amersham, Bethesda Research Labs, New England Biolabs and PL Biochemicals and used as recommended by their suppliers.

(e) Transductions

Lysates of P1Cm1, clr100 and P1*vir* were prepared by heat induction of lysogenic cells (Miller, 1972). Transductions were performed as described by Miller (1972).

(f) Matings

Matings using F′ and Hfr strains were conducted as described by Miller (1972).

(g) Curing of transposon

Elimination of the Tn*10* transposon was accomplished via selection on Tcs media (Maloy and Nunn, 1981).

(h) Transformations

Preparation of competent cells for transformation by pUC plasmid and M13 RF DNA (Hanahan, 1983; Cohen et al., 1972) was modified by using 50 mM $CaCl_2$ and cell harvest at A_{550nm} 0.550–0.720 (0.800–0.890 for JM109). Transformation by M13 RF DNA was as described by Hanahan (1983) and Cohen et al. (1972) except that the heat-shocked cells and DNA were added to B-broth top agar, IPTG, Xgal, and 0.3 ml of host cells grown to an A_{550nm} of 0.720–1.200 (JM109 to 1.200–1.8). No antibiotic amplification period was required for RF DNA. Transformation of each strain were repeated five or more times.

(i) RF and plasmid DNA preparations

pUC plasmid DNA was prepared by inoculation to an A_{550nm} of 0.050 of 2 × YT medium plus Ap with an overnight culture of the plasmid-carrying host strain. The culture was shaken 8–13 h at 37°C before cell harvest. Cm amplification was not required of the multicopy pUC plasmid. RF DNA was prepared by inoculation of 2 × YT medium to an A_{550nm} of 0.05. At A_{550nm} 0.300–0.420, phage supernatant was added to an moi of 10:1, and the culture was shaken 8–13 h at 37°C. DNA was extracted by the Birnboim and Doly method (1979) and purified on CsCl gradients as described by Messing (1983).

(j) Marker tests

The restriction-minus and modification-plus phenotypes were tested by plating 0.1 ml of overnight cultures resuspended in 1XA buffer plus 0.01 M $MgSO_4$ on B-broth plates. 10 μl of the appropriate phage λ*b2c* dilutions (either K-12 modified by two serial propagation cycles through JM101 or K-12 unmodified by two serial propagation cycles through HB101) were spotted on the plates. Plaques were counted after overnight incubation at 37°C. To confirm the modification-plus phenotype, an HB101 λ*b2c* lysate (K-12 unmodified phage) was propagated in the questioned strain for two serial cycles with dilutions of the resultant phage lysate plated on JM101 and HB101. The HB101 lysate was used as a control. No difference in titer was correlated with a modification-plus phenotype.

Phage M13mp10a containing amber mutations in genes I and II (Messing and Vieira, 1982) and M13mp10w with no amber mutations (Norrander

et al., 1983) were tested for the presence of the *su*II suppressor and the F episome that possesses mutation *lacI*^{q} and deletion *lacZ*Δ*M15*. The presence of the *su*II suppressor in F$^-$ strains was confirmed by infection with amber phage T4amN130-N82. Tests confirming the *lacI*^{q} and *lacZ*Δ*M15* deletions via blue plaque production required media supplemented with Xgal and IPTG as described (Messing et al., 1977).

Phage f2 tested for the presence of the *traD36* mutation on the F episome. Unlike M13, the *traD* gene product is required for phage f2 propagation (Achtman et al., 1971). Phage f2's inability to infect an F′ strain confirmed presence of the NIH-recommended *traD36* mutation on the F′.

The *recA* mutation was tested by streaking cells on M9 minimal plates and irradiating with a wavelength of 254 nm for 90 s at a distance of 22.5 cm (handheld UV lamp model UVGL-25 from UVP, Inc. of San Gabriel, CA). As a control, half the agar plate was masked with a paper card during UV exposure. Cells were then incubated overnight at 37°C. UV-resistant growth indicated absence of the *recA* mutation.

The *dam* and *dcm* mutations in GM48 and JM110 were confirmed by propagating pUC plasmids or M13 phage in these strains for at least two overnight culture cycles, isolating the DNA as described above, and cleaving the DNA with either *Mbo*I or *Eco*RII. *Mbo*I cleavage correlated with the *dam* mutation and *Eco*RII cleavage with the *dcm* mutation. *Sau*3A cleavage served as a control.

(k) Construction of JM105

Spontaneous Sm^R mutants of strains SK1592 were selected for on minimal media plates containing high concentrations (500 μg/ml) of Sm. A chromosomal Δ(*lac-proAB*) deletion was introduced into SK1592*rpsL* by crossing with SL10 and selected for by growth in the presence of Sm, 5-fluorocytosine, and proline. The F′ episome, carrying mutations *traD36*, *proAB*, and *lacI*^{q}*Z*Δ*M15*, was transferred to SK1592-*rpsL*-Δ(*lac-proAB*) by mating with JM101. The resulting strain was called JM105 and tested for markers as described above.

(l) Construction of JM106 and JM107

RecA$^+$, Tc^R derivatives of DH1 were obtained by transducing DH1 with P1Cm1*clr*-100 propagated in TD1. The desired transductants, DH1-Tn*10*-*recA*$^+$, were selected by growth on Tc and nalidixic acid, resistance to UV irradiation, and screened for Cm sensitivity.

The Δ(*lac-proAB*) chromosomal deletion was introduced into DH1-Tn*10*-*recA*$^+$ by mating with SL10 and selected for by growth on minimal media plates containing nalidixic acid, 5-fluorocytosine, and proline. Positive progeny were further tested for resistance to UV irradiation and production of white colonies on MacConkey plates or B-broth plates plus Xgal and IPTG.

The *recA*$^+$, Δ(*lac-proAB*), *gyrA96* progeny were tested for retention of the Hsd$^-$ and Su$^+$ (suppressor-plus) phenotypes. Correct progeny were then cured of the Tn*10* transposon by selection on fusaric acid medium. This strain, *recA*$^+$, Δ(*lac-proAB*), *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, was called JM106. JM106(F$^-$) was mated with JM101(F′), and the F′ transfer confirmed by blue plaque production when cells were infected with M13mp-10amber. The final strain was designated JM107.

(m) Construction of JM108 and JM109

The RecA$^-$ phenotype was introduced into JM106 by transducing with P1*vir* propagated on JC10240 (*recA*$^-$, *srlC*::Tn*10*). Progeny were selected for by growth on Tc, proline, nalidixic acid, and 5-fluorocytosine, and screened for inability to grow following exposure to UV light. Further tests affirmed the Hsd$^-$ and Su$^+$ phenotypes and demonstrated white colonies on MacConkey plates and B-broth plates plus Xgal and IPTG.

Positive progeny were cured of the Tn*10* as before and named JM108. JM108 was mated with JM101; progeny were tested for the presence of the F′. The end product (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, λ$^-$, Δ(*lac-proAB*), [F′, *traD36*, *proAB*, *lacI*^{q}*Z*Δ*M15*] was called JM109.

(n) Construction of JM110

Spontaneous Sm^R in GM48 was selected for by plating on minimal plates plus Sm. Inability to

grow without leucine
desired phenotypes.
lished in GM48-*rpsL*
selecting for on m
leucine, 5-fluorocytos
Correct genotype c
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Xgal and IPTG, and
JM110 was the result
proAB) with JM101.
affirmed the F′ prese

(o) Generation of clo

M13UC RF DNA
units each of *Sst*I an
Next, 0.12 vol. of 80
NH_4·acetate and 2
added; after vortexi
15 min, the DNA wa
9000 × *g* for 5 min. T
with cold 70% etha
vacuum. It was resus
(50 mM Tris·HCl
DTT), 8 units of Ex
incubated at 37°C f
removed at 1-min inter
2 μl of 10 × Exo VII
pH 7, 80 mM EDTA
each containing the
time period, were th
Exo VII was added
incubated at 37°C fo
15 min. Next, 1.5 μl
fragment DNA poly
solution of dATP, dC
and incubated at RT
of DNA) aliquot were
(250 mM Tris·HCl p
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DNA ligase and the
with H_2O. After a 3-
was precipitated as d
resuspended in 40 μ
Tris·HCl, pH 7.5,
were used to transfo
Template preparat
electrophoresis, and d

JM107

DH1 were obtained
1*clr*-100 propagated
ctants, DH1-Tn*10*-
on Tc and nalidixic
on, and screened for

nal deletion was in-
+ by mating with
rowth on minimal
lixic acid, 5-fluor-
progeny were further
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s on MacConkey
gal and IPTG.
*rA*96 progeny were
and Su+ (suppres-
progeny were then
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?44, *relA*1, was call-
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esignated JM107.

JM109

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*rA*96, *thi*, *hsdR*17,
[F', *traD*36, *proAB*,
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was selected for
is Sm. Inability to

grow without leucine or threonine confirmed the desired phenotypes. The Δ(*lac-proAB*) was established in GM48-*rpsL* by mating with SL10 and selecting for on minimal plates plus proline, leucine, 5-fluorocytosine and Sm.

Correct genotype confirmation was by screening for white colony production on B-broth plates plus Xgal and IPTG, and by lysis with T4amN130-N82. JM110 was the result of mating GM48-*rpsL*-Δ(*lac-proAB*) with JM101. Infection by M13mp10amber affirmed the F' presence.

(o) Generation of clones for sequencing

M13UC RF DNA (2 μg) was digested with 10 units each of *Sst*I and *Bam*HI for 1.5 h at 37°C. Next, 0.12 vol. of 80 mM EDTA, 0.4 vol. of 5 M NH_4 · acetate and 2 vols. of isopropanol were added; after vortexing and RT incubation for 15 min, the DNA was pelleted by centrifugation at 9000 × *g* for 5 min. The pellet was carefully washed with cold 70% ethanol–water and dried under vacuum. It was resuspended in 40 μl Exo III buffer (50 mM Tris · HCl pH 8, 5 mM $MgCl_2$, 1 mM DTT), 8 units of Exo III were added, and it was incubated at 37°C for 20 min, with 2 μl aliquots removed at 1-min intervals to a tube on ice containing 2 μl of 10 × Exo VII buffer (500 mM K · phosphate, pH 7, 80 mM EDTA, 10 mM DTT). Two tubes, each containing the pooled aliquots from a 10-min time period, were thus generated. Then 0.1 unit of Exo VII was added to each, and the tubes were incubated at 37°C for 45 min, and then at 70°C for 15 min. Next, 1.5 μl 0.2 M $MgCl_2$, 0,5 units large fragment DNA polymerase, and 1 μl of an 8-mM solution of dATP, dCTP, dGTP, TTP were added and incubated at RT for 30 min. To a 5-μl (200 ng of DNA) aliquot were added 5 μl 10 × ligation buffer (250 mM Tris · HCl pH 7.5, 100 mM $MgCl_2$, 25 mM hexamine cobalt chloride, and 5 mM spermidine), 5 μl 10 mM ATP, 2.5 μl 0.1 M DTT, and 2 units DNA ligase and the volume was adjusted to 50 μl with H_2O. After a 3-h incubation at RT, the DNA was precipitated as described above. The pellet was resuspended in 40 μl STE (10 mM NaCl, 10 mM Tris · HCl, pH 7.5, and 1 mM EDTA) and 10 μl were used to transform JM105.

Template preparation, sequencing reaction, gel electrophoresis, and data analysis were as described (Carlson and Messing, 1984; Messing, 1983; Larson and Messing, 1983).

RESULTS AND DISCUSSION

(a) *E. coli* hosts

(1) Conjugation mutants

The male-specific *E. coli* bacteriophage M13 requires an F episome for infection of host cells. Current NIH guidelines regarding the use of recombinant DNA discourage the use of *E. coli* strains carrying conjugation proficient plasmids like the F' episome (Federal Register, 1980). Since the *tra* operon controls conjugation (Achtman et al., 1971), *tra* mutations have been isolated on F'*lac* DNA episomes to develop conjugation deficiencies that still allow infection by M13. M13 vector utility is also based on proper α-complementation between the phage and host β-galactosidase gene. A host strain containing the Δ(*lac-proAB*) deletion on the chromosome and an F'*lac* possessing the *tra* mutation and *lac*ΔM15 deletion was constructed and named JM101 (Messing 1979). JM101 has a suppressor that permitted growth of M13mp7, 8, 9, 10, and 11 phage that contain amber mutations in genes I and II (Messing et al., 1981; Messing and Vieira, 1982).

(2) Restriction mutants

A restrictionless host strain that facilitated cloning of unmodified DNA was constructed and called JM105. As described in MATERIALS AND METHODS, section k, the Δ(*lac-proAB*) chromosomal deletion was introduced into SK1592 via an Hfr cross. Although conjugation of F' with the *traD*36 mutation was reduced by a factor of 10^{-5}, the leaky mutation allowed conjugation at a reduced rate. Therefore, JM101 was used as a donor for the F' *traD*36Δ*proAB lacI*^{q}ZΔM15 episome in mating experiments by using the complementation of proline as selection and drug resistance as counterselection. The resulting strain did not contain the *supE* mutation of JM101, so it permitted growth of wild-type M13mp10, 11, 18, and 19, but not of the amber mutants. This provides selection for transferring inserts from an M13 amber phage into a wild-type

M13 vector and for obtaining M13 recombinants with inserts in the opposite orientation (Carlson and Messing, 1984). Since JM105 is $r^- m^+$, any unmodified DNA cloned directly into wild-type M13 vectors and propagated in JM105 is modified but not restricted. The relevant markers have been tested as shown in Table II.

(3) Recombination mutants

E. coli K-12 restriction was not the only cause for reduced efficiency in cloning larger DNA fragments (> 2000 bp) into M13; another source of instability was recombining sequences. Since *recA* mutations reduce recombination, a host with a *recA* mutation would be useful. A new *recA*, $r^- m^+$, *su*II host for all

TABLE II

Marker tests

(a) Testing for the Hsd^- and Su^+ phenotypes.

λb2c propagated in modifying JM101 or unmodifying HB101 for two serial cycles was spotted on lawns of questioned strains and incubated at 37°C overnight. The JM101-modified and restricted *λb2c* was able to efficiently infect $r^+ m^+$ strains, while HB101 unmodified and unrestricted *λb2c* phage was destroyed in $r^+ m^+$ strains. Amber phage T4amN130-N82 was spotted upon lawns of the strains in question and incubated at 37°C overnight. Only strains possessing the *su*II suppressor gene support growth of the amber phage.

Phage	Phage dilution	Number of plaques on *E. coli* strain:			
		r^+m^+ JM101	r^-m^+ JM105	r^-m^+ JM106	r^-m^- JM108
λb2c propagated in	10^{-2}	40	lysis	lysis	lysis
HB101 (r^-m^-)	10^{-4}	0	lysis	lysis	lysis
(1×10^9 pfu/ml)	10^{-6}	0	8	18	5
λb2c propagated in	10^{-2}	lysis	lysis	lysis	lysis
JM101 (r^+m^+)	10^{-4}	40	lysis	lysis	lysis
(1×10^8 pfu/ml)	10^{-6}	0	0	1	0
T4amN130-N82 propagated in JM101 (1×10^{10} pfu/ml)	10^{-2}	lysis	0	lysis	lysis

(b) Testing for F′, *traD*36, *lacI*^{q}, *lacZ*ΔM15, and for Su^+, Hsd, Rec^-, and Gyr phenotypes.

Infection of plate lawns by spotted dilutions of amber phage M13mp11 + Xgal and +/− IPTG demonstrated presence of the *su*II suppressor gene and the need for IPTG induction of the *lacZ* gene for proper blue plaque production. Phage f2's infectious incapability indicated presence of the *traD*36 mutation in the host strain. Tests for r^- and m^- were as given above in Table IIa. Plates of freshly streaked cells were subjected to UV irradiation to test for the RecA phenotype. Strain growth on plates containing nalidixic acid affirmed the presence of the *gyr* mutation.

Test	Plaques on *E. coli* strain[a]			
	JM101	JM105	JM107	JM109
Infection with M13mp11 amber				
+ Xgal + IPTG	blue	0	blue	blue
+ Xgal alone	clear	0	clear	clear
Infection with f2	0	0	0	0
Test for r^-	−	+	+	+
Test for m^-	−	−	−	−
Growth after UV exposure[a]	+	+	+	−
Growth on nalidixic acid[a]	0	0	+	+

[a] Two bottom lines refer to bacterial growth.

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because it possess
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Clark, 1978) to p
region producing
F′ from JM101, th
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transduction of JM
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four DH1 derivati
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$r^- m^+$ and *su*II p
have been tested f
traD mutations (

(4) Applications

These new str
M13mp and pUC
and JM108 may
libraries, because
DNA can prevent
could be useful
(Helfman et al.,

TABLE III

Transformation effici

Method	
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Method	
$CaCl_2$ Hanahan	

[a] Transformation wi
et al., 1972) with the

M13 vectors was thus constructed. Strain DH1, developed for high transformation efficiencies (Hanahan, 1983), was selected as the initial strain because it possessed a *recA*, *hsdR*17, *supE*44 genotype and high transformation efficiency. The *recA* mutation was transduced to *recA*$^+$ (Csonka and Clark, 1978) to permit deletion of the Δ(*lac-proAB*) region producing JM106. After introduction of the F′ from JM101, the resultant strain JM107 could be used in the same manner as JM101 for infection by amber or wild-type M13 or by the pUC plasmids. P1 transduction of JM106 restored the *recA*1 mutation and produced strain JM108. The introduction of JM101's episome into JM108 produced JM109. All four DH1 derivative strains, JM106, JM107, JM108, and JM109, have been screened for the correct r$^-$m$^+$ and *su*II phenotype, and JM107 and JM109 have been tested for the ΔM15 deletion, the *lacI*^{q} and *traD* mutations (Table II).

(4) Applications

These new strains broaden applications of the M13mp and pUC plasmid vector systems. JM106 and JM108 may prove useful as hosts for cosmid libraries, because deletion of the chromosomal *lac* DNA can prevent background hybridization. JM109 could be useful as the host for cDNA libraries (Helfman et al., 1983; Heidecker and Messing, 1983) and for examining the expression of mutant proteins in *E. coli*. Transformation efficiencies of all strains have been tested by the transformation protocols of Cohen et al. (1972) and Hanahan (1983). All strains approximated the efficiencies of standards DH1 and 71–18, with JM107 and JM109 proving to be slightly higher (Table III). Higher transformation efficiencies have been reported (Hanahan, 1983) and may be possible for these new strains. This work attempted only to ensure that under defined transformation conditions the new strains gave the same transformation efficiencies as the parental strains.

JM109 has proven useful by virtue of its *recA*1 mutation. Plasmids form multimers when propagated in *recA*$^+$ strains like JM83 (Bedbrook and Ausubel, 1976). JM109 maintains pUC species of a unique size whether monomer or multimer. The *recA*1 mutation destroys the mechanisms for the recombination and/or replication events that produce the multimers.

Although it is not possible to predict whether large fragments cloned into M13 and grown in JM109 will experience fewer deletions than when propagated in JM101, the following observations have been made. When a 4.5-kb fragment of the maize-controlling element activator (Ac) was cloned in M13 and propagated in JM107, deletions were found to extend from the Ac sequences into M13 sequences near the

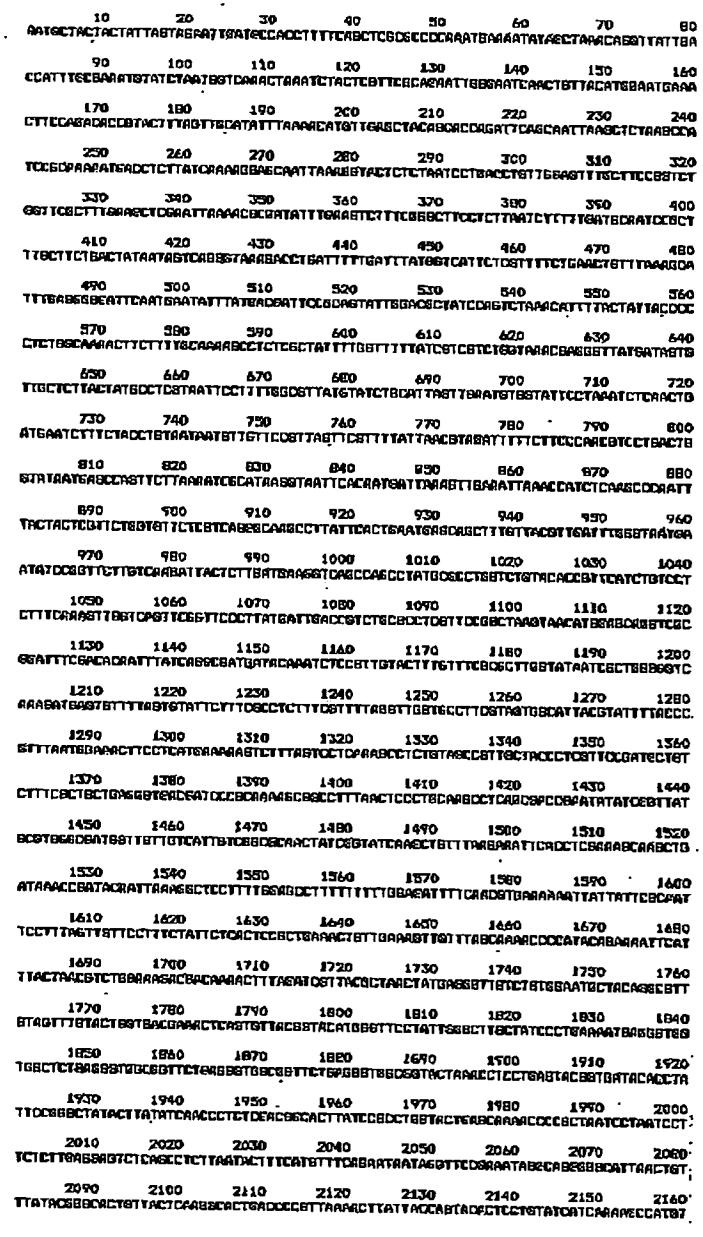
TABLE III

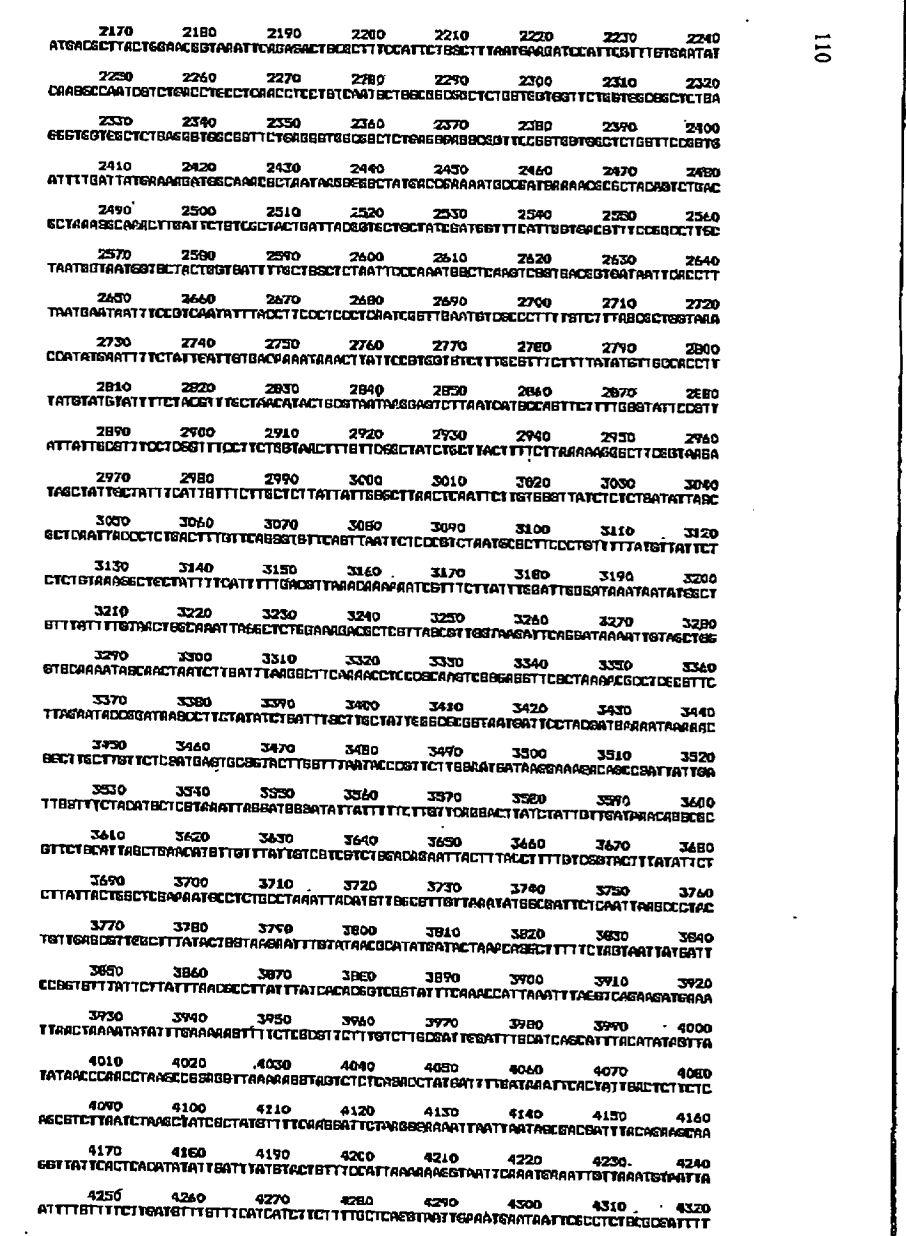
Transformation efficiencies of pUC18 and M13 RF DNAs

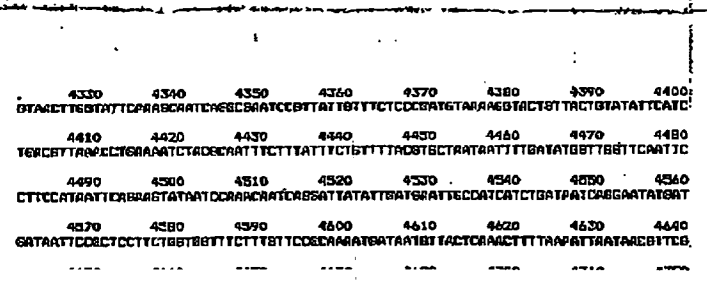
Method	Transformants per μg pUC18 DNA[a] employing *E. coli* recipient strain:			
	DH1	JM105	JM107	JM109
$CaCl_2$	4.2×10^6	8.2×10^6	5.3×10^6	1.0×10^6
Hanahan	6.3×10^6	3.9×10^5	1.4×10^7	1.2×10^7

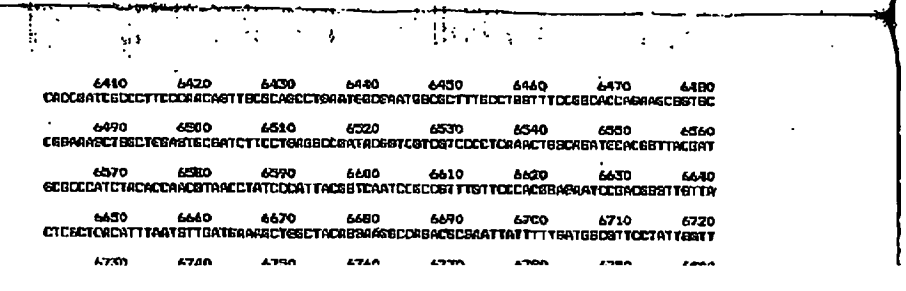
Method	Transformants per μg M13 RF DNA[a] employing *E. coli* recipient strain:			
	71–18	JM105	JM107	JM109
$CaCl_2$	7.3×10^5	3.9×10^5	2.6×10^5	3.4×10^5
Hanahan	3.0×10^5	1.2×10^5	2.0×10^6	1.9×10^6

[a] Transformation with the plasmid or M13 RF DNA into specified host strains, as to compare the traditional $CaCl_2$ method (Cohen et al., 1972) with the Hanahan (1983) protocol.









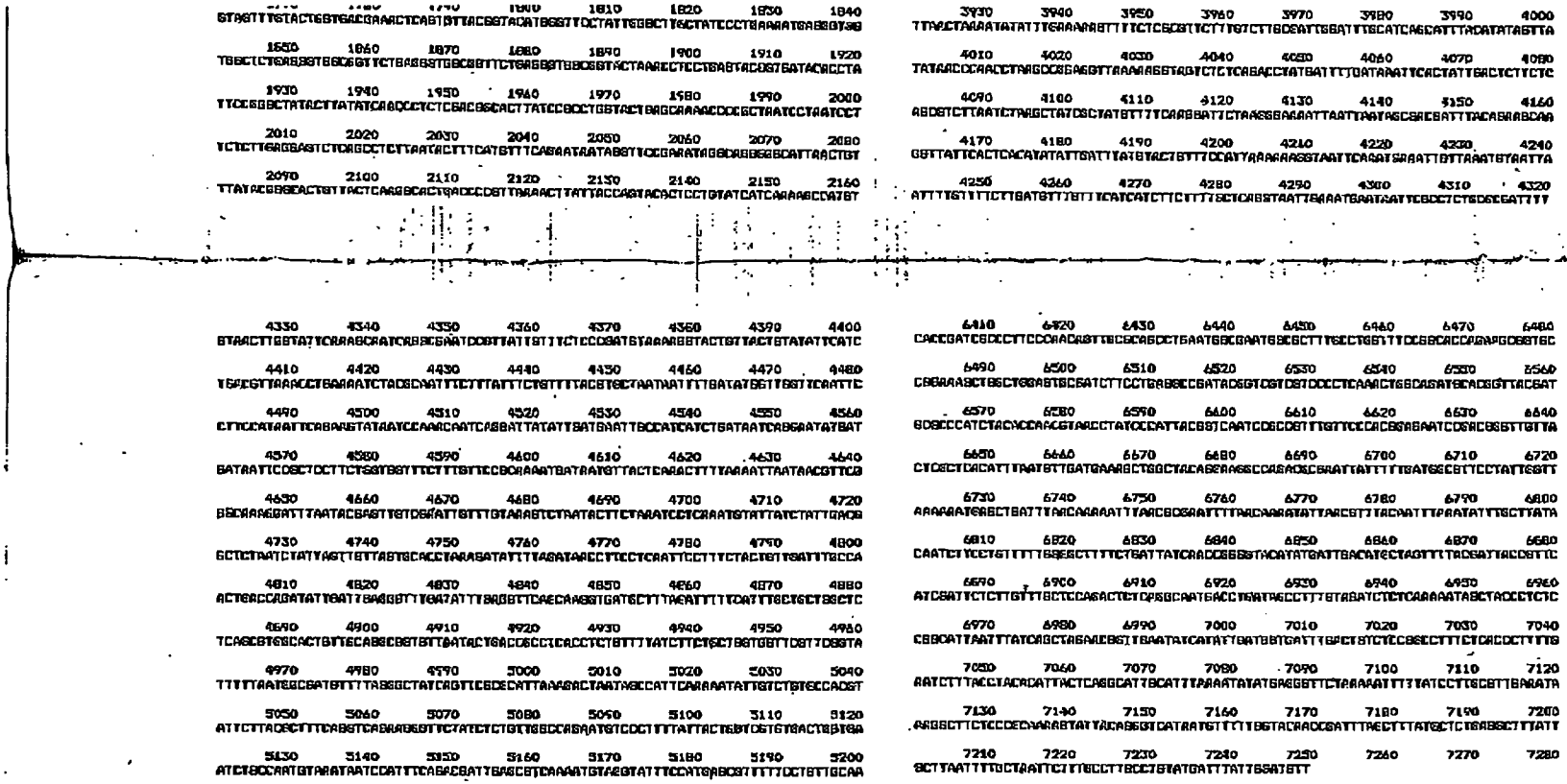


Fig. 1A. The nucleotide sequence of M13mp18. The sequence has been compiled as described in RESULTS, section b, and entered into an Apple II computer. Using the programs described earlier (Larson and Messing, 1983), the sequence has been printed out in the single-strand form using the original *Hin*cII site as reference point. This strand also represents the (+) or message strand. Numbers correspond to bases aligned with the last digit.

NOTE ADDED IN PROOFS:

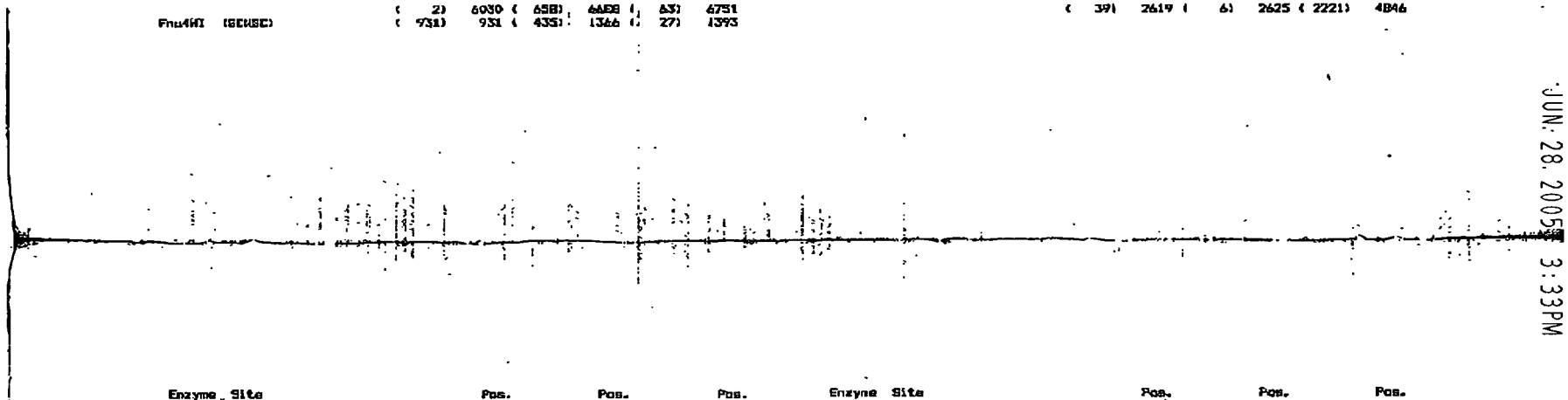
Fig. 1 was modified in proofs because after our paper went into press, we learned from a publication by Dotto and Zinder [Nature 311 (1984) 279–280] that the M13mp phage vectors contain an altered gene II product. They used marker rescue experiments to characterize a G to T substitution at position 6125 of the M13 wild-type sequence (6967 in Fig. 1A), leading to a methionine-to-isoleucine change in the gene II protein (codon 40). The altered gene II protein is expressed at normal wild-type levels in M13mp infected cells, but compensates for the disruption of domain B of the M13 *ori* region. The presented M13 sequence has been changed accordingly.

Enzyme	Site	Pos.	Pos.	Pos.
AccIA	(GTCGAC)	(6263) 6263		
AhaIIC	(GGCGCC)	(6000) 6000		
AluI	(AGCT)	(39) 39 (24) 63 (140) 203		
		(26) 229 (104) 333 (600) 933		
		(554) 1487 (291) 1516 (1446) 2962		
		(313) 3275 (336) 3611 (484) 4095		
		(1330) 5425 (204) 5629 (331) 5960		
		(93) 6053 (64) 6117 (95) 6212		
		(25) 6237 (45) 6282 (93) 6375		
		(111) 6486 (180) 6666 (63) 6729		
		(220) 6949 (27) 6976 (201) 7177		
AvaIA	(CCCGGG)	(6246) 6246		
AvaIC	(CTCGAG)	(5824) 5824		
AvaIIA	(GGACC)	(5913) 5913		
BalI	(TGGCCA)	(5079) 5079		
BamHI	(GGATCC)	(6251) 6251		
BanIA	(GGCAGCC)	(5676) 5676 (454) 6130 (334) 6464		
BanIB	(GGTACC)	(6242) 6242		
BanIC	(GGCGCC)	(6000) 6000		
BanID	(GGTGCC)	(1248) 1248 (5228) 6476		
BanIIB	(GAGCTC)	(6236) 6236		
BanIID	(GGGCTC)	(5642) 5642		
BbVIA	(GCAGC)	(931) 931 (4604) 5535 (516) 6051		
		(303) 6354 (73) 6427		
BbVIB	(GCTGC)	(1366) 1366 (1154) 2520 (611) 3131		
		(1739) 4870 (1052) 5922		
BglI	(GCCNNNNNGGC)	(6430) 6430		
BglII	(AGATCT)	(6934) 6934		
ClaI	(ATCGAT)	(2526) 2526 (4355) 6881		
DdeI	(CTNAG)	(233) 233 (865) 1098 (272) 1370		
		(46) 1416 (367) 1783 (63) 1846		
		(139) 1861 (15) 1876 (24) 1900		
		(72) 1972 (42) 2014 (303) 2317		
		(15) 2332 (15) 2347 (15) 2362		
		(998) 3360 (652) 4012 (27) 4039		
		(39) 4078 (14) 4092 (28) 4120		
		(160) 4280 (600) 4880 (381) 5261		
		(672) 5933 (575) 6508 (399) 6907		
		(153) 7060 (128) 7188		
DraI	(TTTAAA)	(189) 189 (283) 472 (4149) 4621		
		(2162) 6783 (290) 7073		
EcoRI	(GAATTC)	(6230) 6230		
EcoRIIA	(CCAGG)	(5940) 5940 (196) 6136		
EcoRIIB	(CCTGG)	(1013) 1013 (952) 1965 (4032) 5997		
		(330) 6327 (127) 6454		
FnuDII	(CGCG)	(43) 43 (304) 347 (771) 1118		
		(57) 1175 (1290) 2465 (889) 3354		
		(54) 3408 (190) 3598 (353) 3951		
		(361) 4312 (681) 4993 (495) 5488		
		(28) 5512 (20) 5532 (496) 6028		
		(2) 6030 (658) 6688 (63) 6751		
Fnu4HI	(GCNGC)	(931) 931 (435) 1366 (27) 1393		

Enzyme	Site	Pos.	Pos.	Pos.
Fnu4HI	(GCNGC)	(891) 2284 (3) 2287 (24) 2311		
		(45) 2336 (164) 2520 (611) 3131		
		(1739) 4870 (629) 5499 (14) 5513		
		(22) 5535 (387) 5922 (129) 6051		
		(303) 6354 (73) 6427		
FokIA	(GGATG)	(3546) 3546 (3697) 7243		
FokIB	(CATCC)	(239) 239 (6121) 6360		
HaeIIA	(GGCGCC)	(6000) 6000		
HaeIIB	(AGCGCC)	(5588) 5588 (8) 5596		
HaeIIC	(GGCGCT)	(6445) 6445		
HaeIID	(AGCGCT)	(2709) 2709 (329) 3038		
HaeIII	(GGCC)	(1395) 1395 (849) 2244 (309) 2553		
		(2527) 5080 (158) 5238 (106) 5344		
		(69) 5413 (311) 5724 (214) 5938		
		(98) 6036 (257) 6293 (102) 6395		
		(117) 6512 (169) 6681 (341) 7022		
HgaIA	(GACGC)	(526) 526 (16371) 2163 (315) 2478		
		(758) 3236 (3450) 6686		
HgaIB	(GCGTC)	(4082) 4082 (1075) 5157		
HgiaIA	(GTGCAC)	(4742) 4742		
HgiaIB	(GAGCTC)	(6236) 6236		
HgiaIC	(GTGCTC)	(5464) 5464		
HhaI	(GCGC)	(44) 44 (966) 1010 (74) 1084		
		(92) 1176 (293) 1469 (725) 2194		
		(272) 2466 (244) 2710 (329) 3039		
		(56) 3095 (312) 3407 (190) 3597		
		(714) 4311 (683) 4994 (495) 5489		
		(13) 5502 (9) 5511 (22) 5533		
		(26) 5559 (8) 5567 (434) 6001		
		(28) 6029 (65) 6094 (331) 6425		
		(21) 6446 (115) 6561		
HincIIA	(GTCGAC)	(6263) 6263		
HindIII	(AAGCTT)	(6281) 6281		
HinfI	(GANTC)	(136) 136 (80) 216 (2741) 490		
		(21) 511 (2121) 723 (12873) 2010		
		(486) 2496 (348) 2844 (413) 3257		
		(160) 3417 (324) 3741 (96) 3837		
		(234) 4071 (45) 4116 (232) 4348		
		(771) 5119 (209) 5328 (46) 5374		
		(63) 5437 (328) 5765 (22) 5787		
		(2533) 8040 (221) 6261 (362) 6623		
		(261) 6884 (19) 6903 (137) 7040		
MspAI	(CCGG)	(3141) 314 (6511) 965 (129) 1094		
		(8291) 1923 (454) 2377 (18) 2395		
		(1561) 2551 (818) 3369 (472) 3841		
		(1761) 4017 (15961) 5613 (545) 6158		
		(89) 6247 (215) 6462 (18) 6480		
		(357) 6837 (123) 6960 (60) 7020		
NphIA	(GGTGA)	(1375) 1375 (398) 1773 (135) 1908		
		(489) 2397 (144) 2541 (39) 2580		
		(39) 2619 (6) 2625 (2221) 4846		

Enzyme	Site	Pos.	Pos.	Pos.
NphIA	(GGTGA)	(270) 5116 (589) 5705 (241) 5946		
		(33) 5979 (1025) 7004		
NphIB	(TCACC)	(1502) 1502 (1132) 2634 (2288) 4922		
		(2108) 7030		

Enzyme	Site	Pos.	Pos.	Pos.
RsaI	(GTAC)	(143) 1164 (604) 1768 (27) 1795		
		(93) 1888 (16) 1904 (65) 1969		
		(163) 2132 (1334) 3466 (201) 3667		
		(522) 4189 (190) 4379 (1004) 5383		



Enzyme	Site	Pos.		Pos.		Pos.
HphIA	(GGTGA)	(270) 5116	(589) 5705	(241) 5946		
		(33) 5979	(1025) 7004			
HphIB	(TCACC)	(1502) 1502	(1132) 2634	(2288) 4922		
		(2108) 7030				
KpnI	(GGTACC)	(6242) 6242				
MboI	(GATC)	(1381) 1381	(332) 1713	(507) 2220		
		(4032) 6252	(153) 6405	(96) 6501		
		(434) 6935				
MboIIA	(GAAGA)	(2217) 2217	(1694) 3911	(2479) 6390		
MboIIB	(TCTTC)	(781) 781	(3293) 4074	(196) 4270		
		(666) 4936	(318) 5254	(332) 5586		
		(917) 6503	(301) 6804			
MnlIA	(CCTC)	(254) 254	(119) 373	(187) 560		
		(27) 587	(68) 655	(383) 1038		
		(49) 1087	(143) 1230	(66) 1296		
		(21) 1317	(8) 1325	(19) 1344		
		(71) 1415	(90) 1505	(391) 1896		
		(48) 1944	(75) 2019	(243) 2262		
		(6) 2268	(404) 2672	(4) 2676		
		(217) 2893	(158) 3051	(269) 3320		
		(31) 3351	(351) 3702	(604) 4306		
		(391) 4697	(74) 4771	(149) 4920		
		(5) 4925	(421) 5346	(69) 5415		
		(265) 5680	(340) 6020	(235) 6255		
		(277) 6532	(423) 6955			
MnlIB	(GAGG)	(484) 484	(141) 625	(747) 1372		
		(359) 1731	(102) 1833	(15) 1848		
		(15) 1863	(15) 1878	(129) 2007		
		(312) 2319	(15) 2334	(15) 2349		
		(15) 2364	(4) 2368	(967) 3335		
		(685) 4020	(799) 4819	(15) 4834		
		(612) 5446	(947) 6393	(117) 6510		
		(575) 7086	(105) 7190			
MstI	(TGCGCA)	(6424) 6424				
MstII	(CCTNAGG)	(6307) 6307				
NaeI	(GCCGGC)	(5612) 5612				
NarI	(GGCGCC)	(6000) 6000				
NciIA	(CCCGG)	(6246) 6246				
NciIB	(CCGGG)	(1923) 1923	(4324) 6247	(590) 6837		
NdeI	(CATATG)	(2722) 2722	(1080) 3802	(3043) 6845		
NlaIII	(CATG)	(149) 149	(47) 196	(910) 1106		
		(193) 1299	(499) 1798	(235) 2033		
		(123) 2156	(698) 2854	(677) 3531		
		(87) 3618	(99) 3717	(1461) 5178		
		(1043) 6221	(55) 6276	(580) 6856		
NlaIV	(GGNNCC)	(1061) 1061	(187) 1248	(292) 1540		
		(10) 1550	(252) 1802	(248) 2050		
		(323) 2373	(18) 2391	(3252) 5643		
		(12) 5655	(21) 5676	(190) 5866		
		(134) 6000	(130) 6130	(112) 6242		
		(9) 6251	(213) 6464	(12) 6476		
PstI	(CTGCAG)	(6269) 6269				
PvuI	(CGATCG)	(6404) 6404				
PvuII	(CAGCTG)	(5959) 5959	(93) 6052	(322) 6374		
RsaI	(GTAC)	(173) 173	(107) 280	(741) 1021		

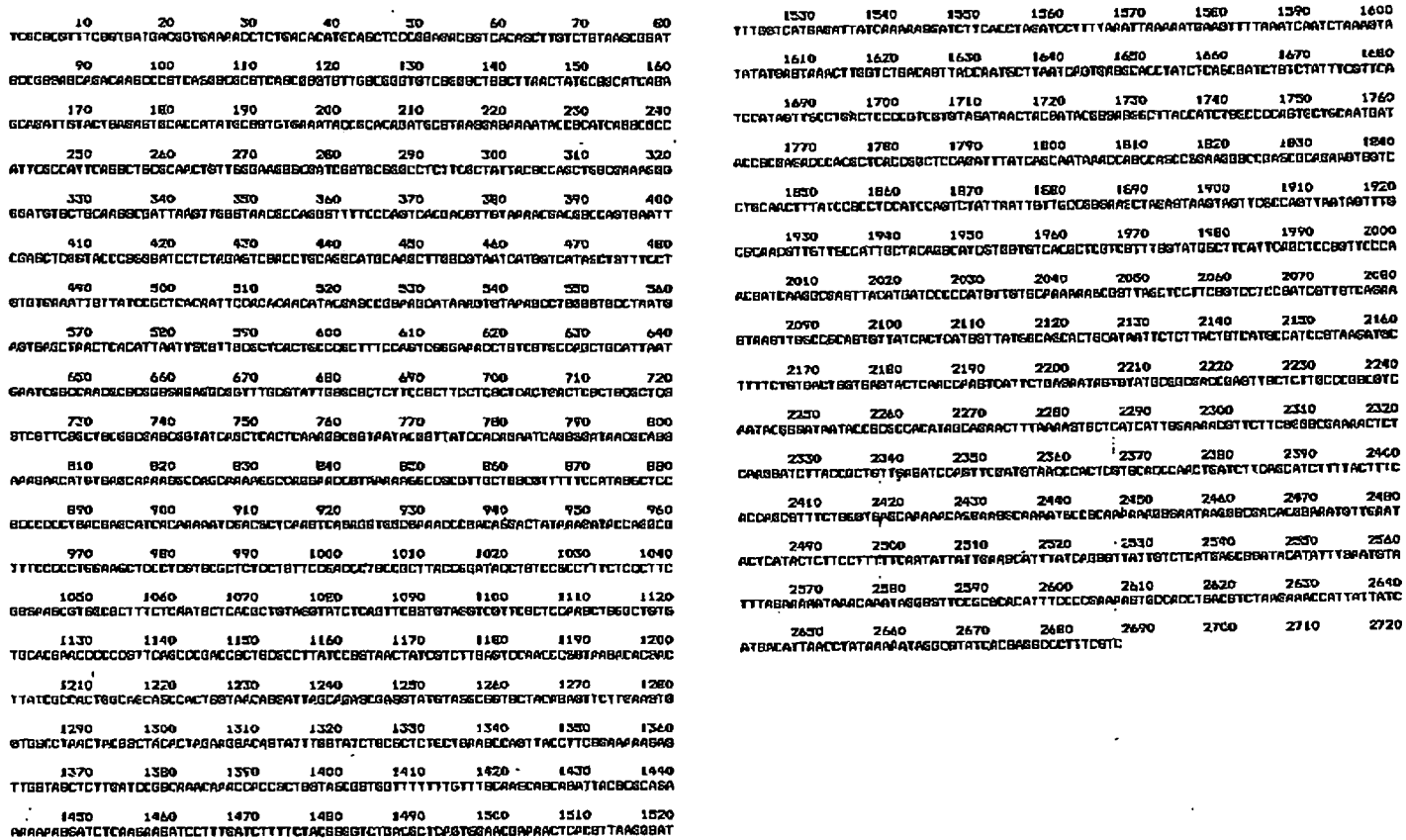
Enzyme	Site	Pos.		Pos.		Pos.
RsaI	(GTAC)	(143) 1164	(604) 1768	(27) 1795		
		(93) 1888	(16) 1904	(65) 1969		
		(163) 2132	(1334) 3466	(201) 3667		
		(522) 4189	(190) 4379	(1004) 5383		
		(102) 5485	(758) 6243	(599) 6842		
		(322) 7164				
SacI	(GAGCTC)	(6236) 6236				
SalI	(GTCGAC)	(6263) 6263				
Sau96I	(GGNCC)	(5723) 5723	(190) 5913	(24) 5937		
		(458) 6395				
ScrFI	(CCNGG)	(1013) 1013	(910) 1923	(42) 1965		
		(3973) 5940	(57) 5997	(139) 6136		
		(110) 6246	(1) 6247	(80) 6327		
		(127) 6454	(383) 6837			
SfaNIA	(GATGC)	(251) 25	(363) 388	(965) 1353		
		(3496) 4849	(1696) 6545	(131) 6508		
SfaNIB	(GCATC)	(3978) 3978				
SmaI	(CCCGGG)	(6246) 6246				
SnaBI	(TACGTA)	(1267) 1267				
SphI	(GCATGC)	(6275) 6275				
TaqI	(TCGA)	(336) 336	(790) 1126	(381) 1507		
		(441) 1948	(579) 2527	(927) 3454		
		(239) 3693	(971) 4664	(1018) 5682		
		(552) 6234	(30) 6264	(618) 6882		
XbaI	(TCTAGA)	(6257) 6257				
XhoIIA	(GGATCC)	(6251) 6251				
XhoIIB	(AGATCC)	(2219) 2219				
XhoIID	(ABATCT)	(6934) 6934				
XmnI	(GAANNNNTTC)	(357) 357	(2288) 2645			

These enzymes did not appear.....

AatII	AccIB	AccIC	AccID	AhaIIA
AhaIIB	AhaIID	ApaI	AvaIB	AvaID
AvaIIB	BanIIA	BanIIC	BclI	BssHII
BstXI	BsteII	EcoRV	HgiaID	HincIIB
HincIIC	HincIID	HpaI	MluI	NcoI
NotI	NruI	NsiI	SacII	ScaI
StuI	TthIII	XhoI	XhoIIC	XmaIII

Fig. 1B. Restriction sites of M13mp18. The position (Pos.) and distances (in parentheses) between the neighbouring restriction sites are specified in nucleotides, as compiled and printed out employing the restriction mapping program. (See legend to Fig. 1A).

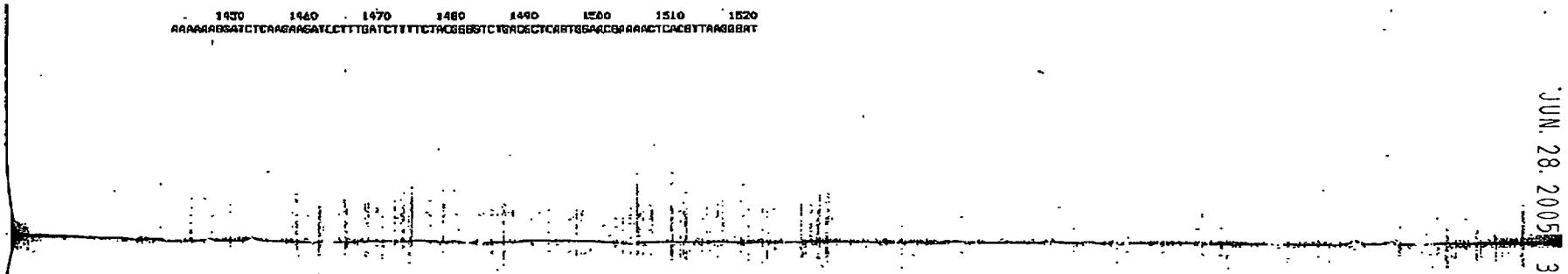
(A)



(B)

ENZYME	SITE	POS.	POS.	POS.
ACCIA	(GTCGAC)	(429) 429		
ACVIA	(GGGCGC)	(235) 235		
ACVIB	(GACGTC)	(2235) 2235		
ACVID	(GACGTC)	(2617) 2617		
ALUI	(AGCT)	(43) 43 (19) 62 (245) 307		
		(961) 403 (45) 448 (22) 470		
		(95) 365 (64) 429 (118) 747		

ENZYME	SITE	POS.	POS.	POS.
HPAII	(CCGG)	(111) 529 (4693) 5013 (147) 5160		
		(261) 1186 (190) 1376 (404) 1780		
		(341) 1814 (67) 1881 (1103) 1991		
		(2421) 2233		
HPHIIA	(GGTGA)	(12) 12 (9) 21 (2152) 2173		
		(241) 2414		
HPHIB	(TCACC)	(1550) 1550 (227) 1777 (622) 2399		



(B)

ENZYME	SITE		POS.		POS.		POS.
ACCIA	(GTCGAC)	(429)	429				
ACYIA	(GGCGCC)	(235)	235				
ACYIB	(GGCGYC)	(2235)	2235				
ACYID	(GPCGYC)	(2617)	2617				
ALUI	(AGCT)	(43)	43	(19)	62	(245)	307
		(96)	403	(45)	448	(22)	470
		(95)	565	(64)	629	(118)	747
		(226)	973	(136)	1109	(257)	1366
		(521)	1887	(100)	1987	(63)	2050
ASUI	(GGNCC)	(286)	286	(1534)	1820		
AVAIA	(CCGGGG)	(412)	412				
AVAIIB	(GGTCC)	(1637)	1837	(222)	2059		
BAMHI	(GGATCC)	(417)	417				
BBVIA	(GCAGC)	(41)	41	(1172)	1213	(3)	1216
		(206)	1422	(694)	2116		
BBVIB	(GCTGC)	(254)	254	(73)	327	(303)	630
		(81)	711	(18)	729	(419)	1148
		(602)	1750				
BBLI	(GCGNNNNNGGC)	(245)	245	(1568)	1813		
BDEIA	(CTRAG)	(2622)	2622				
BDEIB	(CTCAG)	(1081)	1081	(409)	1490	(166)	1656
BDEIC	(CTGAG)	(171)	171	(2025)	2196		
BCORI	(GNATTC)	(396)	396				
BCORIIA	(CCAGG)	(354)	354	(479)	833	(121)	954
BCORIIB	(CCTGG)	(545)	545	(422)	967		
BMUDII	(GDGC)	(2)	2	(2)	4	(103)	107
		(545)	652	(2)	654	(198)	852
		(281)	1433	(330)	1763	(493)	2256
		(332)	2588				
BMU4HIA	(GCGGC)	(650)	650	(155)	1005	(1084)	2089
		(351)	2440				
BMU4HIB	(GCGGC)	(150)	150	(582)	732	(1479)	2211
BAEIA	(ABGCCA)	(819)	819	(11)	830		
BAEIA	(TGGCCT)	(1282)	1282				
BAEIIA	(GGCGCC)	(235)	235				
BAEIIC	(GCGCGC)	(680)	680	(370)	1050		
BAEIII	(GGCC)	(287)	287	(102)	389	(257)	646
		(174)	820	(11)	831	(18)	849
		(434)	1283	(459)	1741	(80)	1821
		(267)	2088				
BGAIA	(GAGGC)	(908)	908	(578)	1486		
BGAIB	(GCGTC)	(108)	108	(2128)	2236		
BGIAIA	(GTGCAC)	(177)	177	(943)	1120	(1246)	2366
BGIAIB	(GAGCTC)	(402)	402				
BGIAIC	(GTGCTC)	(2281)	2281				
BHAI	(GCGC)	(3)	3	(103)	106	(130)	236
		(21)	257	(331)	588	(65)	653
		(28)	681	(33)	714	(270)	984
		(67)	1051	(100)	1151	(174)	1325
		(109)	1434	(393)	1827	(93)	1920
		(337)	2257	(332)	2589		
HINCIIA	(GTCGAC)	(429)	429				
HINDIII	(AAGCTT)	(447)	447				
HINFIA	(GAATC)	(641)	641	(140)	781		
HINFIB	(GACTC)	(706)	706	(988)	1694		
HINFIC	(GAGTC)	(427)	427	(750)	1177		
HPAII	(CCGG)	(48)	48	(34)	82	(331)	413

ENZYME	SITE		POS.		POS.		POS.
HPAII	(CCGG)	(111)	324	(489)	1013	(147)	1160
		(26)	1186	(190)	1376	(404)	1780
		(34)	1814	(67)	1881	(110)	1991
		(242)	2233				
HPHIIA	(GGTGA)	(12)	12	(9)	21	(2152)	2173
		(241)	2414				
HPHIB	(TCACC)	(1530)	1530	(227)	1777	(622)	2399
KPNI	(GGTACC)	(408)	408				
MBOI	(GATC)	(277)	277	(141)	418	(955)	1373
		(75)	1448	(11)	1459	(8)	1467
		(78)	1545	(12)	1557	(105)	1662
		(341)	2003	(18)	2021	(961)	2067
		(258)	2325	(17)	2342	(36)	2378
MBOIIA	(GAAGA)	(1456)	1456				
MBOIIB	(TCTTC)	(291)	291	(394)	685	(862)	1547
		(755)	2302	(78)	2380	(109)	2489
MLUIA	(CCTC)	(29)	29	(260)	289	(132)	421
		(274)	695	(283)	978	(878)	1856
		(206)	2062				
MLUIB	(GAGG)	(662)	662	(259)	921	(324)	1245
		(400)	1645	(81)	1726	(947)	2673
MSTI	(TGCGCA)	(256)	256	(1663)	1919		
PSTI	(CTGCAG)	(935)	935				
PVUI	(CGATCG)	(276)	276	(1790)	2066		
PVUII	(CAGCTG)	(306)	306	(322)	628		
RSAI	(GTAC)	(168)	168	(241)	409	(1769)	2178
SACI	(GAGCTC)	(402)	402				
SALI	(GTCGAC)	(429)	429				
SFANIA	(GATGC)	(78)	78	(130)	208	(1948)	2156
SFANIB	(GCATC)	(153)	153	(76)	229	(665)	894
		(1052)	1946	(440)	2386		
SMAI	(CCCGGG)	(412)	412				
SPHI	(GCATGC)	(441)	441				
TAQI	(TCGA)	(400)	400	(30)	430	(476)	906
		(1444)	2350				
XBAI	(TCTAGA)	(423)	423				
XHOIIA	(GRATCC)	(417)	417				
XHOIIB	(AGATCC)	(1438)	1438	(98)	1536	(785)	2341
XHOIIC	(GGATCT)	(1447)	1447	(97)	1544	(780)	2324
XOEI	(CATATG)	(183)	183				
XORI	(GGCGCC)	(235)	235				
XSTI	(TGCGCA)	(256)	256	(1663)	1919		

THESE ENZYMES DID NOT APPEAR.....				
ACCIB	ACCIC	ACCID	ACYIC	AEUI
AVAIB	AVAIC	AVAID	AVAIIA	AVAIII
AVRII	BALI	BCLI	BGLII	BSTEIIA
BSTEIIB	BSTEIIC	BSTEIID	CLAI	DDEID
HAEIA	HAEIC	HAEIIB	HAEIID	HGIAID
HINCIIB	HINCIIC	HINCIID	HINFID	HPAI
BACII	XHOI	XHOIIB	XMAIII	NDOI

Fig. 2. The nucleotide sequence and restriction sites of pUC19. (A) The sequence has been compiled as described and entered into an Apple II computer. The information derived from sequencing pUC6 (Fig. 4) was used to make corrections via the programs described earlier (Larson and Messing, 1983). Both the *bla* and the *lac* α-peptide gene products are read from the same strand. Since all coordinates of the pBR322 sequence refer to the opposite strand, the single-stranded DNA with the polarity published by Sutcliffe (1979) is given. The message strand is obtained with the program that produces the reverse complement of pUC19 (referred to as pUC19V). (B) Restriction site coordinates are as in Fig. 1B.

M13 origin of replication (Pohlman, R. and Messing, J., unpubl.). It should be noted that the Ac element contains numerous direct and indirect repeats in regions of low and high G + C contents (Pohlman et al., 1984). When the 4.5-kb *Pst*I-*Bam*HI Ac fragment is cloned into M13 and grown in JM109, stable recombinants have been recovered and no deletions have been detected over numerous generations (not shown).

(5) Methylation

Other *E. coli* mutations useful for recombinant DNA amplification are DNA methylation deficiencies. For example, *Mbo*I and *Bcl*I can cleave DNA propagated in a *dam*⁻ *E. coli* strain while *Eco*RII restriction requires the absence of the *dcm* product (for review, see Roberts, 1983). If DNA is propagated in *E. coli* strains lacking the A and C methylases, it is unmodified and can be cleaved by *Mbo*I, *Bcl*I, and *Eco*RII. GM48 contains these mutations, but lacks the *Δ(lac-proAB)* deletion and the F′ *traD36 proAB lacI*^{q}*ZΔ*M15 episome required for M13mp and pUC vector use. Strain JM110 was developed to be *dam*⁻ *dcm*⁻ by introduction of the *Δ(lac-proAB)* deletion and JM101 episome into GM48. The *dam* and *dcm* mutations have been tested as described in MATERIALS AND METHODS, section **j** (not shown).

(b) M13 phage vectors

Since many specific constructions depend on the knowledge of the vector restriction map, the nucleotide sequences of M13mp18 and pUC19 have been compiled and reprinted here. The wild-type sequence of M13 has been determined by Van Wezenbeek et al. (1980). M13's unique *Hin*cII site was used as the sequence reference point. Following this reference, the C in position 3 has been converted to a T to eliminate the *Hin*cII site, the G (2220) converted to an A to eliminate the *Bam*HI site, and the C (6917) converted to a T to eliminate the *Acc*I site and introduce the *Bgl*II site (positions are for wild-type M13) (Messing et al., 1981). The *lac Hin*dII fragment has been inserted into the *Bsu*I site at position 5868 (Messing et al., 1977). The *lac* sequence has been compiled from the *lacI* gene (Farabaugh, 1978), the *lacZpo* region (Dickson et al., 1975), and the *lacZ* gene sequences (Kalnins et al., 1983). M13mp18 and M13mp19 lack the double amber mutation that M13mp10a and M13mp11a had (Messing and Vieira, 1982) but do possess two complementary polylinker regions in the *lacZ* gene (Norrander et al., 1983). The junctions of *lac* DNA and M13 DNA were as predicted from the restriction sites used for cloning the *lac Hin*dII fragment into the *Bsu*I site at position 5868. The junction sequence at the *lacZ* gene led to an early ochre termination codon that produced a *lac* α fragment of 168 amino acid residues; 18 of them represent the polylinker region. The resulting sequence of M13mp18 is presented in Fig. 1.

(c) The pUC plasmids

(1) Construction

The *lac Hae*II fragment inserted into pBR322 produced a shorter α peptide than that in M13 (Ruther, 1980), yet active. The pBR322 sequence has been modified by removing the *Eco*RI-*Pvu*II fragment containing the Tc resistance gene via a fill-in reaction and blunt-end ligation. The predicted regeneration of the *Eco*RI site failed to occur when sequencing data revealed that the deletion extends across nucleotides 4355-1-2072. Similar findings were reported by Rubin and Spradling (1983) and Stragier, P. (personal communication). Restriction sites were removed from the intermediate plasmid in the following way. EMS mutagenesis resulted in a GC to AT transition in the *Pst*I site at position 3610 (positions are for pBR322) (Vieira and Messing, 1982). Hydroxylamine treatment converted another GC to AT in the *Hin*cII site at position 3911. The *Acc*I site was eliminated by BAL31 digestion of nucleotides 2210–2250. The *lac* sequence was inserted at the *Hae*II site at position 2352. The *lac* sequence was oriented in the same direction as the *bla* gene coding for β-lactamase. The fusion of the *lacZ* sequence to the pBR322 DNA at the *Hae*II site at position 2352 resulted in an α peptide of 107 amino acid residues, 19 encoded by the polylinker region at residue 5. Translation was terminated by the UAG termination codon, which was suppressed in the *supE* strains. In *supE* strains the peptide was 15 amino acids longer and terminated by an UGA codon. These small fusion peptides were very unstable in *E. coli* and detectable only through the highly sensitive complementation test with Xgal. The nucleotide sequence and restriction map for pUC19 are given in Fig. 2.

(2) Sequencing with nev

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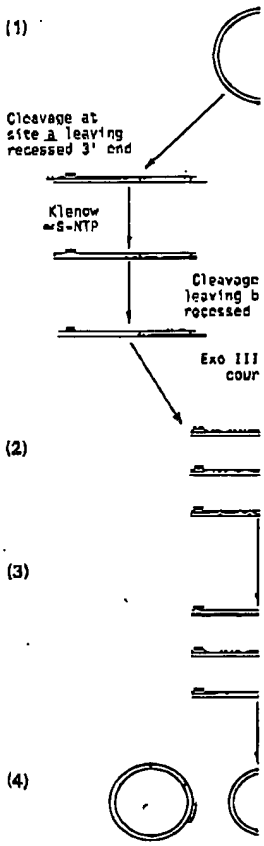

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(2) Sequencing with new method

Since the pUC plasmids have been mutagenized with EMS, HA, and treated with BAL31 before introducing the *lac* DNA into the pBR322 backbone molecule, the pBR322 portion of pUC6 (Vieira and Messing, 1982) had to be resequenced. Earlier sequencing experiments were based on M13 shotgun

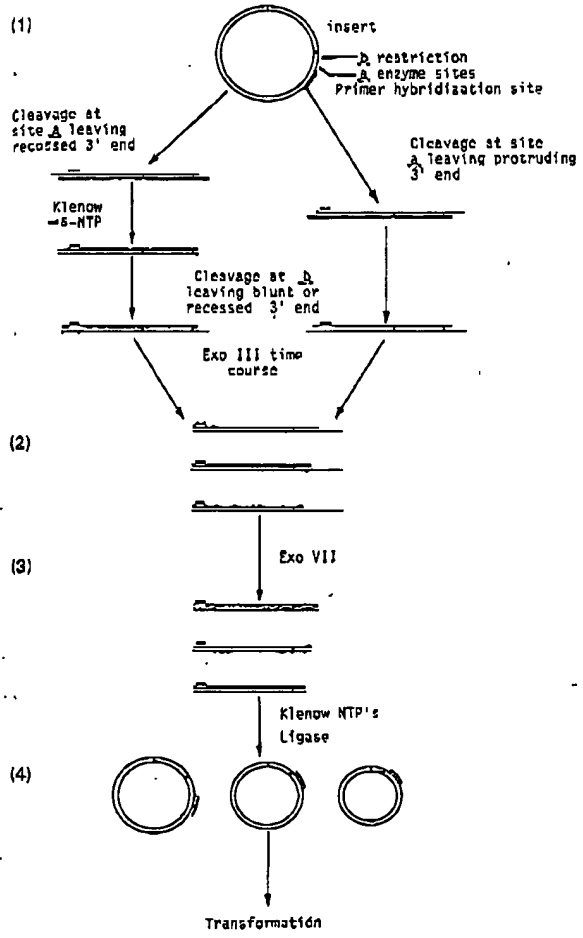

Fig. 3. Method for generating unidirectional deletions. Details of the procedure are as follows: (1) Vector is digested with restriction enzymes a and b. Enzyme a leaves a 4-bp 3′ overhang that is resistant to Exo III and protects the PHS site. Enzyme b leaves a recessed 3′ end that is sensitive to Exo III and exposes the insert to digestion. (2) The DNA is treated with Exo III for 0–20 min and aliquots removed at 1 min intervals. This generated random insert deletions while leaving the PHS intact. (3) Exo VII removed the single-stranded DNA region left by Exo III. (4) The digest was treated with DNA polymerase I to ensure formation of blunt-ended DNA. DNA ligase was added to recircularize the various deletion products, leading to increasingly smaller circles with the PHS in the same position.

sequencing of pUC6 (Halling, S., Abbot, A., Kridl, J. and Messing, J., unpubl.). Because the asymmetric M13 polylinkers (Vieira and Messing, 1982) could be used to make unidirectional deletions, a different approach of generating pUC6 subclones for sequencing was tested. The polylinker permitted cleavage of the pUC plasmid or the M13 RF by two restriction endonucleases, one producing a 3′ protruding end like *Pst*I, the other a 5′ protruding end. Since Exo III was double-strand-specific and required a 3′OH end, the *Pst*I end was not accessible to this enzyme. These features simplified the nonrandom sequencing approach based on *BAL*31 treatment described below (Poncz et al., 1982) and illustrated in Fig. 3. The method included the following steps: (1a) pUC6 was linearized with *Nde*I, and the ends were made flush with the large fragment of DNA polymerase and inserted at the *Hin*cII site of M13mp19 to make M13UC. This produced, between the inserted DNA and the PHS, a unique *Sst*I site proximal to the PHS and a unique *Bam*HI site distal to it. (1b) M13UC RF was digested with *Sst*I and *Bam*HI. *Sst*I leaves a 4-bp 3′ overhang that is resistant to Exo III and protects the PHS from digestion. *Bam*HI leaves a recessed 3′ end that is sensitive to Exo III and exposes the insert to digestion. (2) The DNA was treated with Exo III for 0–20 min. Aliquots were removed at 1-min intervals. This time course generated random insert deletions while leaving the PHS intact. (3) The single-stranded region of DNA left by Exo III treatment was removed with Exo VII. Since only Exo VII is active in the presence of EDTA, addition of Exo III time-course aliquots to a tube containing Exo VII buffer plus EDTA proves a convenient way to stop the Exo III reaction. (4) To ensure formation of blunt-ended DNA, the digest was treated with DNA polymerase I. DNA ligase was added to recircularize the molecules, which were then used to transform JM105. (5) Phage isolated from transformed cells were used for direct gel electrophoresis (Messing, 1983) to determine clones of appropriate size for sequencing (Fig. 4).

The Exo III, Exo VII, polymerase, and ligase reactions were performed sequentially by adjusting reaction buffers. Alternatively, it was possible to protect the PHS from Exo III digestion via S-NTP incorporation by DNA polymerase at a recessed 3′ end proximal to the PHS left by restriction endo-

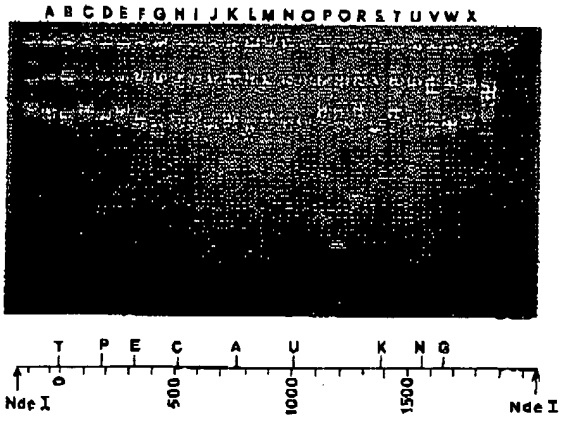

Fig. 4. Mapping of the deletion mutants. Since the position of the PHS is unaltered and all deletions occur only at the opposite end, deletion points are mapped by recombinant phage mobility changes indicated by agarose gel electrophoresis. After exonuclease and ligase treatment, the DNA is transformed into JM105. Plaques are picked from each transformation experiment, grown in small cultures, and supernatant phage used directly for agarose gel electrophoresis as described (Messing, 1983). A picture taken of the agarose gel was used to draw a physical map of the sequenced clones. The first and last lanes (unmarked) contain untreated M13UC1 and M13mp19, respectively; the other lanes are labeled alphabetically and represent individual clones. Nine clones from this gel were used to prepare a template for sequencing as described in MATERIALS AND METHODS, section e. The sequence has been entered into an Apple II computer and analyzed using the programs of Larson and Messing (1983). The deletion points are marked in the map by the agarose-gel-derived clone name. The map has been drawn with reference to the *Nde*I sites used to clone pUC6 into the *Hin*cII site of M13mp19. The nucleotide numbers in the map are taken from the reverse complement of pUC6, referred to as pUC6V.

nuclease digestion (Putney et al., 1981; Vieira, J., unpublished results). Cleavage of a site distal to the PHS was then needed to generate an unprotected recessed 3′ end for Exo III treatment. The consecutive steps are outlined in Fig. 3.

This approach resembles that described by Poncz et al. (1982), but hastens the construction of recombinant M13 phage needed for sequencing. As opposed to bidirectional deletions, the creation of unidirectional deletions precludes the need for recloning DNA fragments. The speed by which recombinants can be obtained resembles that of shotgun cloning. Ordering clones on a physical map is simple (Fig. 4), so the redundancies and gaps typical of shotgun sequencing are avoided. Hence, the following sequencing approach to larger DNA segments is used. Restriction sites present in the polycloning sites of M13mp18 and M13mp19 are used to clone restriction fragments in both orientations. Fragments need to be inserted such that between the PHS and the insert there exist two unique restriction enzyme sites. The restriction enzyme site proximal to the PHS must produce either a 4 bp 3′ overhang or a recessed 3′ end. The other should leave a blunt or recessed 3′ end next to the insert. Each clone pair representing both orientations is then subjected to Exo III and VII treatment. The optimum insert size for nuclease treatment is 2000–5000 bp. Also, the ExoIII unit activity of different commercial preparations can vary, necessitating the calibration of each enzyme lot. This is accomplished by the electrophoresis of DNA samples taken at two time points from an ExoIII reaction on an agarose gel for size analysis.

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Long Terminal Repeat of Friend-MCF Virus Contains the Sequence Responsible for Erythroid Leukemia

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Friend-MCF virus induces erythroid leukemia when injected into newborn NFS mice whereas Moloney virus induces T-cell lymphoma. To identify the portion of Friend-MCF virus responsible for erythroid leukemia induction four *in vitro* recombinant viruses were constructed in which *env* regions or U3 regions of LTR were reciprocally exchanged between Friend-MCF and Moloney viruses. A FrMCF-Mol (LTR) virus whose genome was derived primarily from Friend-MCF virus together with 621 nucleotides of Moloney virus at its 3′ end including the U3 region of LTR was a thymic lymphoma-inducing virus. A Mol-FrMCF (LTR) virus with the genome derived primarily from Moloney virus but 596 nucleotides of Friend-MCF virus information at the same region as FrMCF-Mol (LTR) was an erythroid leukemia-inducing virus. A Mol-FrMCF (env) virus whose genome was derived primarily from Moloney virus but which had 2.8 kbp of Friend MCF at the 3′ end of the *pol* gene including most of the *env* gene with all of gp70 and the N terminal of p15E was a lymphoid leukemia-inducing mink cell focus-inducing virus. FrMCF-Mol (env) virus whose genome was derived primarily from Friend-MCF virus but had 2.7 kbp of Moloney virus at the same region as Mol-FrMCF (env) virus was an erythroid leukemia-inducing ecotropic virus. The Mol-FrMCF (LTR) and Mol-FrMCF (env) viruses induced mixed leukemia of erythroid and lymphoid cells in some mice.

There are two proposed mechanisms of tumorigenesis by murine leukemia viruses (MuLV) both of which seem to be possible rather than alternative: (1) the activation of some cellular gene by insertion of the viral transcription regulatory elements near the cellular sequence, (2) *env* gene or *env*-related gene products may play a role in tumorigenesis by the virus. Oliff and Ruscetti (1983) showed that a 2.4-kbp fragment of the Friend virus genome contains the sequences responsible for Friend murine leukemia virus-induced erythroleukemia, when the 2.4-kbp fragment is inserted into amphotropic MuLV. This fragment encompassed approximately 700 bp from the 3′ end of the Friend virus *pol* gene and 1.7 kbp of the *env* gene including all of the gp70 and the N terminal four-fifths of p15E.

Their recombinant gave only 25% incidence of disease and with a much longer latent period than the starting Fr-MuLV. However, other results obtained from *in vitro* recombinant virus between molecularly cloned oncogenic and nononcogenic virus (Lenz *et al.*, 1984) and between viruses with different pathogenesis (DeGroseillers, 1983) have shown that the LTR has an important role in thymic lymphomagenesis, especially in determining the target tissue of the virus. Chatis *et al.* (1983) showed that a recombinant whose genome is derived primarily from Friend virus but contains the LTR of Moloney virus induces almost exclusively thymic lymphomas, inspite of the presence of Oliff's 2.4-kbp fragment. DeGroseillers *et al.* (1983) also showed that the thymotropism of MuLV is conferred by its LTR

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their sequence data suggested that U3 tandem direct repeat was responsible for this effect. Lenz *et al.* (1984) suggested that the enhancer region sequences are the major determinants of leukemogenicity in leukemogenic virus isolated from AKR mice.

The present study was aimed at determining whether the erythroleukemogenicity was also determined by the LTR similar to thymic lymphoma genicity. Genetic information for cytopathic effects of mink cell focus-inducing (MCF) virus are also described.

MATERIALS AND METHODS

Viruses. Infectious DNA clones (Adachi *et al.*, 1984) of Friend-MCF virus (Ishimoto *et al.*, 1981) were subcloned into pBR322 at the *Eco*RI site and designated Bp-1 and Bp-2. An infectious DNA clone of ecotropic Moloney virus was obtained from Dr. Weinberg of MIT (Hoffmann *et al.*, 1982). It was resubcloned into pBR322 at the *Hin*dIII site in our laboratory and designated Molp-1.

Restriction enzymes and digestion. DNA was digested with 2 U of enzyme/μg DNA under the buffer conditions specified by the manufacturer. For more than one cleavage, a restricted DNA sample was ethanol precipitated, and the pellet was washed in 70% ethanol, air dried, and suspended in the reaction buffer of the second enzyme. Digested DNA was analyzed by electrophoresis at 30 V on 0.7% agarose horizontal slab gels. The standard size marker mixture consisted of DNA fragments ranging from 23.7 to 0.10 kb and was prepared by the product of *Hin*dIII- or *Eco*RI-cleaved λDNA.

Construction of DNA recombinants in vitro. For subcloning, about 10 μg of various subclones were cleaved with restriction endonucleases. The digestion conditions used were those recommended by the suppliers (Takara Shuzo Co. Ltd., Kyoto, Japan and Toyobo Co. Ltd., Osaka, Japan). The desired fragments were separated from 0.7% low-melting-point agarose (Bethesda Research Lab, Md.) gel under the conditions specified by the supplier. These fragments were then ligated to cleaved pBR322 with T4 DNA ligase (Takara Shuzo Co. Ltd. Kyoto) at 12° for 20 h in 10 μl of a solution containing 50 m*M* Tris-hydrochloride (pH 7.5), 10 m*M* $MgCl_2$, 20 m*M* dithiothreitol, and 1 m*M* ATP and used to transform *E. coli* HB101. Colonies were screened by the alkaline lysis rapid isolation method (Birnboim and Doly, 1979). Positive clones were grown in mass culture. Plasmid DNA was extracted and molecularly characterized by excising the insertion with the appropriate restriction endonucleases using agarose or polyacrylamide gel electrophoresis.

Transfection. Viral inserts were cleaved from the subclones with the appropriate restriction endonuclease and separated from 0.7% low-melting-point agarose gel. Viral DNAs separated from pBR322 were ligated with T4 DNA ligase. Ligation was confirmed by the ethidium bromide staining of the gel, most of the DNAs were converted to circular or linear dimers, and several unknown forms of higher molecular weight DNA. The religated DNA was then transfected onto 6-cm plates containing NIH3T3 or SC-1 cells by a modification (Wigler *et al.*, 1979) of the original calcium phosphate precipitation method (Graham and Eb, 1973).

Cells and virus assays. SC-1 cell (Hartley and Rowe, 1975), NIH3T3 (Todaro and Green, 1963), mink lung cell ATCC CCL-64 (Henderson *et al.*, 1974), mink S+L− cells (Peebles, 1975) were grown in heated 5% FCS in the Dulbecco-Vogt modification of EMEM. XC cells were grown in heated 10% calf serum in EMEM.

Ecotropic virus infection of mouse cells was determined by the XC-plaque assay (Rowe *et al.*, 1970). Dualtropic MCF virus assays were performed with the focus assay in the mink S+L− cells or the "focus" in mink lung cells. Phenotypically mixed virus was assayed as described previously (Ishimoto *et al.*, 1977, 1981).

Mice. NFS mice are an inbred strain from an NIH Swiss mouse originally supplied by the animal production section of the NIH. A continuous single line was maintained in our laboratory by sibling

mating. The recovered MCF virus (0.2 ml) (10^4 S+L− mink cell FFU/mouse) harvested from mink cells and 0.2 ml of the recovered ecotropic virus (10^5 XC PFU/mouse) harvested from SC-1 cells were inoculated intraperitoneally into newborn NFS mice.

RESULTS

In Vitro Construction of Recombinant Viral DNAs in Which the LTR Regions Were Reciprocally Exchanged between Ecotropic Moloney and Friend-MCF Vi-

Two restriction endonuclease sites shared by Friend-MCF and Moloney virus were used to construct their *in vitro* recombinant viruses the *Ban*III site located in the p15E portion of the *env* gene and the *Kpn*I site in the R region of the proviral LTR (Fig. 1). The same sites had already been used to construct a recombinant viral DNA between ecotropic Friend and ecotropic Moloney viruses by Chatis *et al.* (1983). The restriction endonuclease *Ban*III is an isoschizomer of *Cla*I which was used by Chatis *et al.*

The LTR region of Moloney virus was first introduced into Friend-MCF virus to learn whether Friend-MCF viruses induce thymic lymphoma when the LTR region of Friend-MCF virus is exchanged with that of ecotropic Moloney virus. This has been previously shown for the ecotropic Friend virus (Chatis *et al.*, 1983). The DNA clone of Moloney virus was digested with restriction endonucleases to produce the 621-bp *Ban*III-*Kpn*I fragment, while the DNA clone of Friend-MCF Bp-1 was digested completely with *Kpn*I and then partially with *Ban*III to yield *Ban*III-*Kpn*I LTR region-free fragments (the *Ban*III-*Kpn*I larger fragment). These fragments were eluted from the low-melting-point agarose gel and ligated with T4 DNA ligase to form a recombinant clone B(m)-3 which was derived primarily from Bp-1 but contained the LTR region of Molp-1. The structure of B(m)p-3 recom-

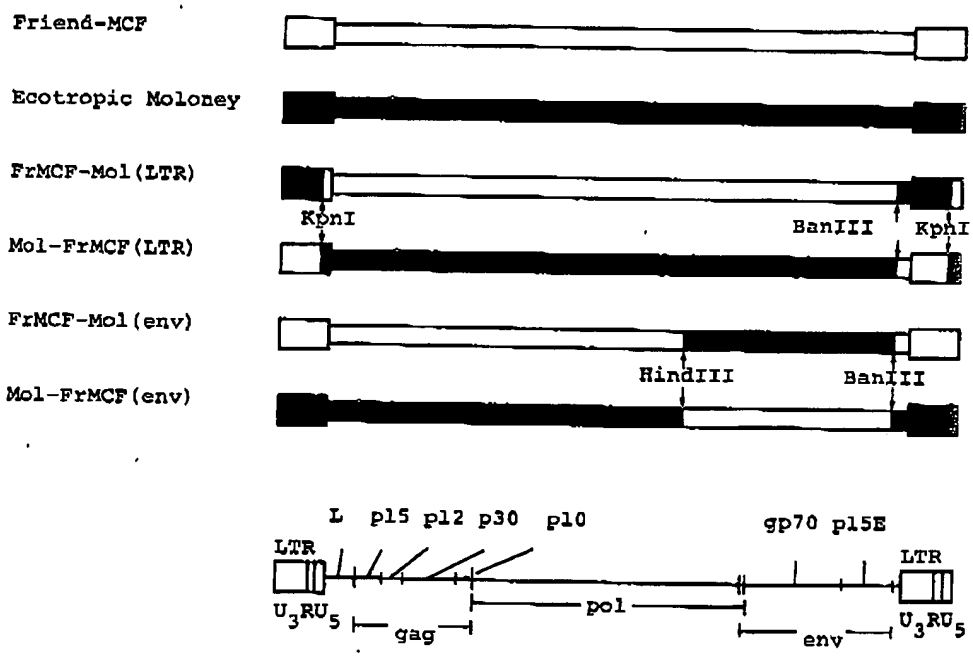

FIG. 1. Nonpermuted form of the parental and recombinant viral DNA genomes. The viral genomes are presented as linear double-stranded molecules flanked at their ends by LTR sequences (boxes). Positions of *gag*, *pol*, and *env* are shown at the bottom. The open areas indicate FrMCF or fragments derived from it, and the solid areas indicate ecotropic Moloney or fragments derived from it. Three restriction endonuclease sites shared by Friend-MCF and Moloney viruses for constructing *in vitro* recombinant viruses are indicated.

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s is exchanged with acrylamide electrophoresis: the *Ban*III–
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ey virus was digested sessed one copy of LTR whereas Bp-1 had
nucleases to produce two. Viral inserts in B(m)p-3 and Ep-2
*Kpn*I fragment, while were excised by *Eco*RI and ligated with
riend-MCF Bp-1 was T4 DNA ligase to obtain a recombinant
with *Kpn*I and then virus designated FrMCF-Mol (LTR) after
II to yield *Ban*III– transfection into the cells.
ree fragments (the Next, we constructed a Moloney virus
fragment). These with the LTR region from Friend-MCF
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and ligated with T4 struction of the virus is a little more
a recombinant clone complicated than that for constructing
rived primarily from B(m)p-3. As shown in Fig. 2 the recom-
the LTR region of binant DNA subclone M(B)p-1 in pBR322
e of B(m)p-3 recom- was constructed through two intermediate
subclones (E+Mol)p-1 and (B+Mol)p-1.
The intermediate subclone (E+Mol)p-1
was obtained by the insertion of a full-
length viral DNA from a Molp-1, *Hin*dIII–
*Hin*dIII fragment, into a *Hin*dIII–*Hin*dIII
fragment of Ep-2, and a clone which was
ligated at the *Hin*dIII site within their
pol gene was selected. Another interme-
diate subclone (B+Mol)p-1 was con-
structed by the insertion of the *Hin*dIII–
*Kpn*I fragment from Molp-1 into the
*Hin*dIII–*Kpn*I fragment of Bp-1. A final
subclone M(B)p-1 was constructed by the
insertion of the *Eco*RI–*Ban*III fragment
from subclone (E+Mol)p-1 into the *Eco*RI–
*Ban*III fragment from (B+Mol)p-1. The
*Hin*dIII–*Hin*dIII fragment was excised
from the M(B)p-1 and ligated with T4
DNA ligase to obtain a recombinant virus
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In Vitro Construction of Recombinant Viral DNA in Which the env Gene Region Was Reciprocally Exchanged between Moloney and Friend-MCF Virus

The *Hin*dIII site located within the *pol* gene and *Ban*III site within the p15E portion of the *env* gene shared by Friend-MCF and Moloney virus were used to construct *in vitro* recombinant viruses whose *env*-gene regions were exchanged reciprocally (Fig. 1).

We first constructed a recombinant designated FrMCF-Mol (env) whose genome was derived primarily from Friend-MCF virus but which had a 2.7-kbp *Hin*dIII–*Ban*III fragment including most of the *env* gene from Moloney virus (Fig. 1). The 2.7-kbp fragment of Moloney virus DNA encompassed approximately 1.0 kbp from the 3′ end of the Moloney virus *pol* gene and 1.9 kbp of the *env* gene including all of the gp70 and the N terminal of p15E. A recombinant DNA subclone (B+E+Mol)p-1 was constructed by subclone Bp-1 and (E+Mol)p-1 and the infectious viral DNA genome was excised with *Eco*RI from subclone (B+E+Mol)p-1 which was derived primarily from Bp-1 and Ep-2 but contained a 2.7-kbp fragment from Molp-1.

To construct a recombinant designated Mol-FrMCF (env) with a genome derived primarily from Moloney but with a *Hin*dIII–*Ban*III fragment from Friend-MCF virus including the *env* gene, we first tried to construct a recombinant subclone (E+B)p-1 in which the full *env*-gene sequence of Friend-MCF virus was connected. This was done since the *env* gene of Friend-MCF virus was split into two subclones DNA Bp-1 and Ep-2 at the *Eco*RI site located within the *env* gene (Fig. 3). The *Hin*dIII–*Ban*III fragment from Friend-MCF virus was approximately 2.3 kbp, and encompassed about 700 bp from the 3′ end of the Friend-MCF *pol* gene and 1.7 kbp of the *env* gene including all the gp70 and the N terminal of p15E. A 2.3-kbp *Hin*dIII–*Ban*III fragment of Friend-MCF virus from subclone (E+B)p-1 and a 6.1-kbp *Ban*III–*Hin*dIII fragment of Moloney virus from Mop-1 were excised and ligated with T4 DNA ligase for transfection into the cells.

Infectivity of in Vitro Recombinant Viral DNA

To recover Friend-MCF, FrMCF-Mol (LTR), and Mol-FrMCF (env) viruses by

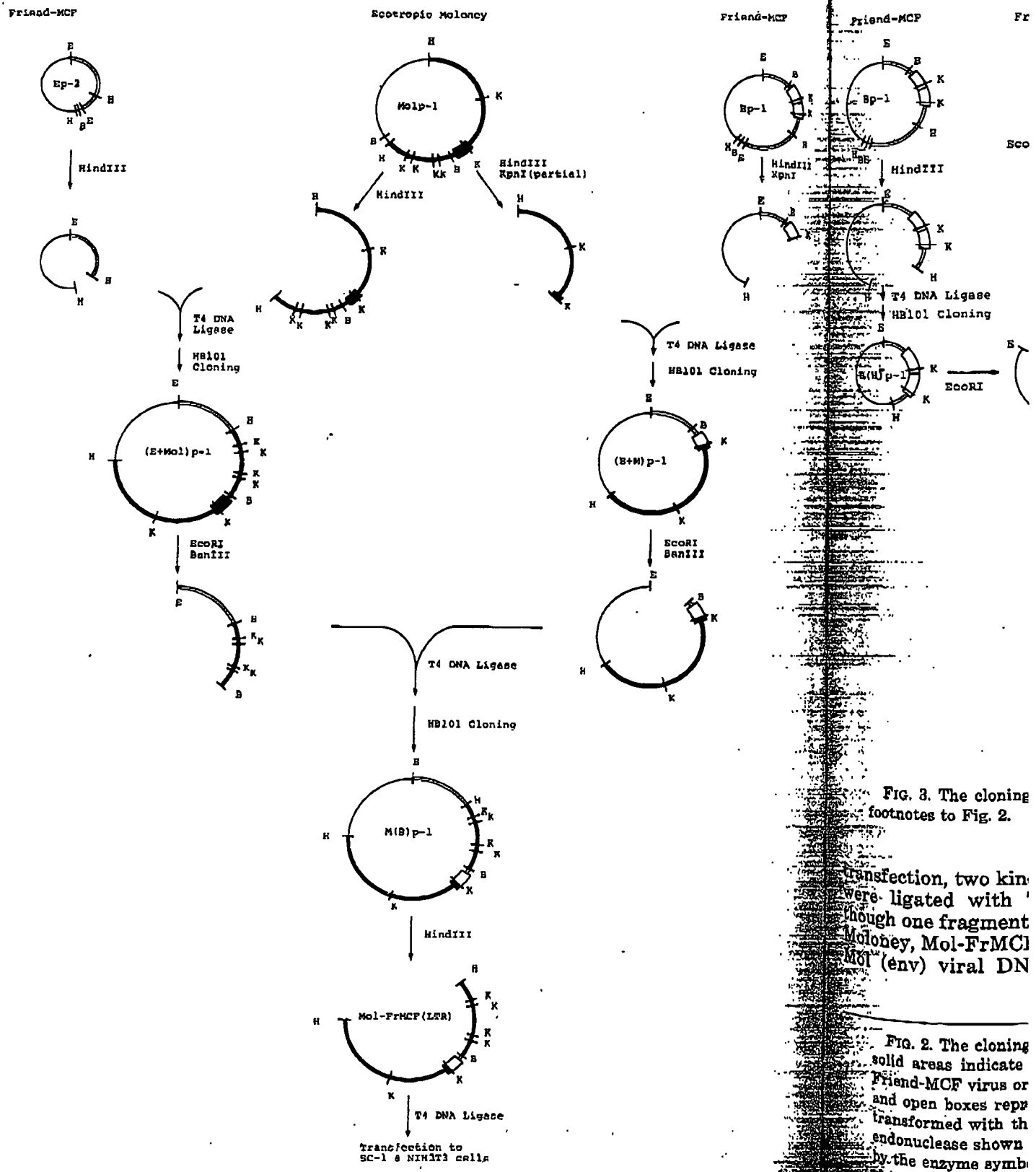

FIG. 3. The cloning
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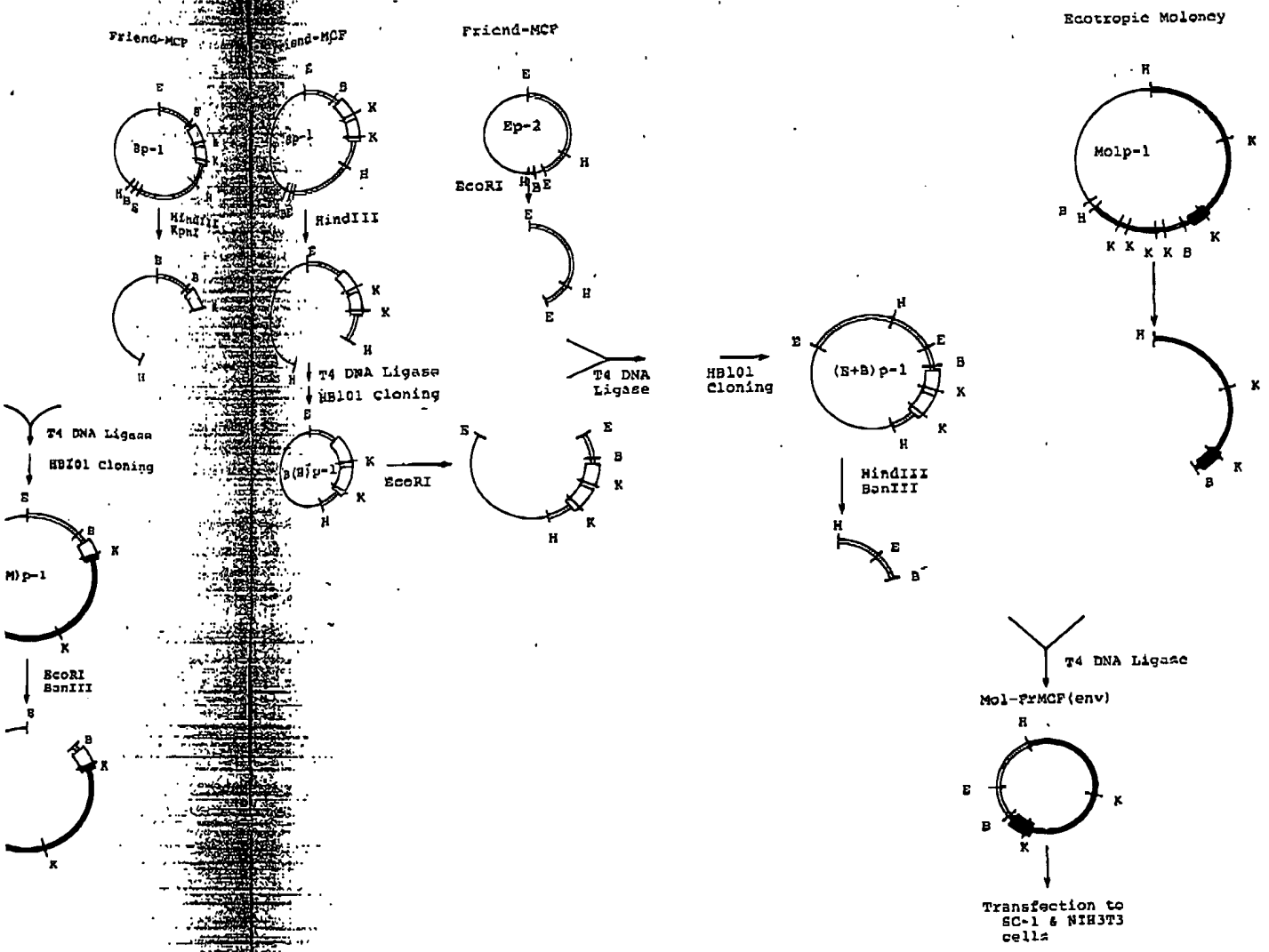

FIG. 3. The cloning strategy used to construct Mol-FrMCF (env) viral DNA recombinant. See footnotes to Fig. 2.

transfection, two kinds of DNA fragments were ligated with T4 DNA ligase. Although one fragment contained full-length Moloney, Mol-FrMCF (LTR), and FrMCF-Mol (env) viral DNAs, the viral DNAs were also ligated with T4 DNA ligase for transfection to create recombinant DNAs containing two LTRs, since the parent DNA has one LTR. Each of the ligated recombinant viral DNAs was transfected

FIG. 2. The cloning strategy used to construct Mol-FrMCF (LTR) viral DNA recombinant. The solid areas indicate Moloney virus or fragments from Moloney virus, the open areas indicate Friend-MCF virus or fragments from Friend-MCF virus, and the lines indicate pBR322. The solid and open boxes represent the LTRs. HB101 cloning means that the *Escherichia coli* HB101 was transformed with the DNA for cloning. The recombinant DNA was cleaved with the restriction endonuclease shown beside the arrow. The restriction endonuclease sites of subclones are shown by the enzyme symbols K, *Kpn*I; B, *Ban*III; H, *Hind*III; E, *Eco*RI.

onto five 6-cm plates containing SC-1 cells and five plates containing NIH3T3 cells. The supernatants of the plates were harvested every 2 days to test for viral production. Viral production was usually observed in the supernatant by 2 weeks after transfection. However, ecotropic viruses were usually detected earlier than MCF viruses. Although SC-1 cells seem less sensitive than NIH3T3 cells for transfection, SC-1 cells were better than NIH3T3 cells for detecting ecotropic viruses by the *in situ* uv-XC test, since the XC plaque was not so clear when uv-XC procedure was tried on the NIH3T3 cells. To detect MCF viruses, the NIH3T3 or SC-1 cells transfected with viral DNA were cultured with mink cells. When MCF viruses appeared, the cytopathogenic changes of mink cells were usually found before getting the assay result from the supernatant. Independent isolates of recombinant viruses were harvested from both SC-1 and NIH3T3 cells, and their oncogenicity and viral properties were examined except for FrMCF-Mol (env) virus. FrMCF-Mol (env) virus was never recovered from SC-1 cells. Two independent isolates of FrMCF-Mol (env) virus were harvested from two dishes of NIH3T3 cells.

Virological Properties of Recovered Recombinant Virus

The recombinant FrMCF-Mol (LTR) and Mol-FrMCF (env) viruses which contained the 2.3-kbp *Hin*dIII-*Ban*III fragment of the Friend-MCF virus were NB-tropic, XC-negative, dualtropic, mink cell focus-inducing viruses as well as parental Friend-MCF virus. Thus it appears that the information for the cytopathic effect of Friend-MCF virus lies in the 2.3-kbp *Hin*III-*Ban*III fragment from Friend-MCF virus.

The recombinant Mol-FrMCF (LTR) and FrMCF-Mol (env) which contained the 2.7-kbp *Hin*dIII-*Ban*III fragment from the Moloney virus were NB-tropic, ecotropic XC-positive viruses as well as parental Moloney virus. The XC-negative Friend-MCF virus was converted to XC-positive FrMCF-Mol (env) virus by the substitution of the *Hin*dIII-*Ban*III fragments with the corresponding region of the Moloney virus.

Oncogenicity of in Vitro Recombinant Viruses

Parental and recombinant viruses obtained from DNA transfections were inoculated ip into newborn NFS mice. The oncogenicity and the latent period of the viruses are shown in Table 1. Friend-MCF viruses induced erythroid leukemia after 2–5 months, as has already been reported by us (Ishimoto *et al.*, 1981). Moloney viruses induced lymphomas after 1.5–3 months as reported by Chatis *et al.* (1983). The latent period for the two viruses is similar, but Moloney virus-induced lymphoma appeared earlier than Friend-MCF virus-induced erythroid leukemia.

FrMCF-Mol (LTR) virus induced lymphomas in the NFS mice. Histologically, the leukemias were typical T-cell lymphomas with involvement of spleen, lymph node, thymus, and liver. No pathological differences were identified among lymphomas induced with Moloney and FrMCF-Mol (LTR) viruses except for the incidence of leukemia and the latent period.

Mol-FrMCF (LTR) virus indueed erythroid leukemia in 160 mice and lymphomas in 5 mice from a total of 184 mice injected. The erythroid mice showed marked hepatosplenomegaly, but no enlargement of thymus or lymph node. Histologically they were typical erythroid leukemia. Histological examination of the five mice with lymphomas revealed their enlarged thymus and lymph nodes to be lymphoid cell tumors, but unexpectedly their enlarged spleens were typical of erythroid leukemia. Thus, the five mice seemed to have mixed leukemia lymphoid and erythroid cells (Fig. 4). Mol-FrMCF (LTR) viruses induced erythroid or mixed leukemia with higher incidence than the parental Friend-MCF virus. The latent period of the recombinant virus was also much shorter than that of the parental virus.

FrMCF-Mol (env) virus induced erythroid leukemias as early and frequent

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TABLE 1

LEUKEMOGENICITY OF VARIOUS RECOMBINANT VIRUSES IN NFS MICE

Virus inoculated (host range)	No. of mice[a] inoculated	Mice with erythroid leukemia		Mice with lymphoid leukemia		Mice with mixed leukemia of lymphoid and erythroid cells	
		No. of leukemic mice (incidence %)	Mean latent period (days)	No. of leukemic mice (incidence %)	Mean latent period (days)	No. of leukemic mice (incidence %)	Mean latent period (days)
Parental							
Friend-MCF (dualtropic)	49	38 (78)	116	0		0	
Moloney (ecotropic)	18	0		18 (100)	58	0	
Recombinant							
FrMCF-Mol (LTR) (dualtropic)	141	0		14 (10)	125[b]	0	
Mol-FrMCF (LTR) (ecotropic)	184	160 (87)	60	0		5 (3)	65
FrMCF-Mol (env) (ecotropic)	87	71 (82)	56	0		0	
Mol-FrMCF (env) (dualtropic)	105	0		12 (11)	88[c]	2 (2)	80

[a] Mice observed more than 10 months after inoculation.
[b] Except three leukemic mice with latent periods of 8, 8, and 9 months.
[c] Except a leukemic mouse with latent period of 9 months.

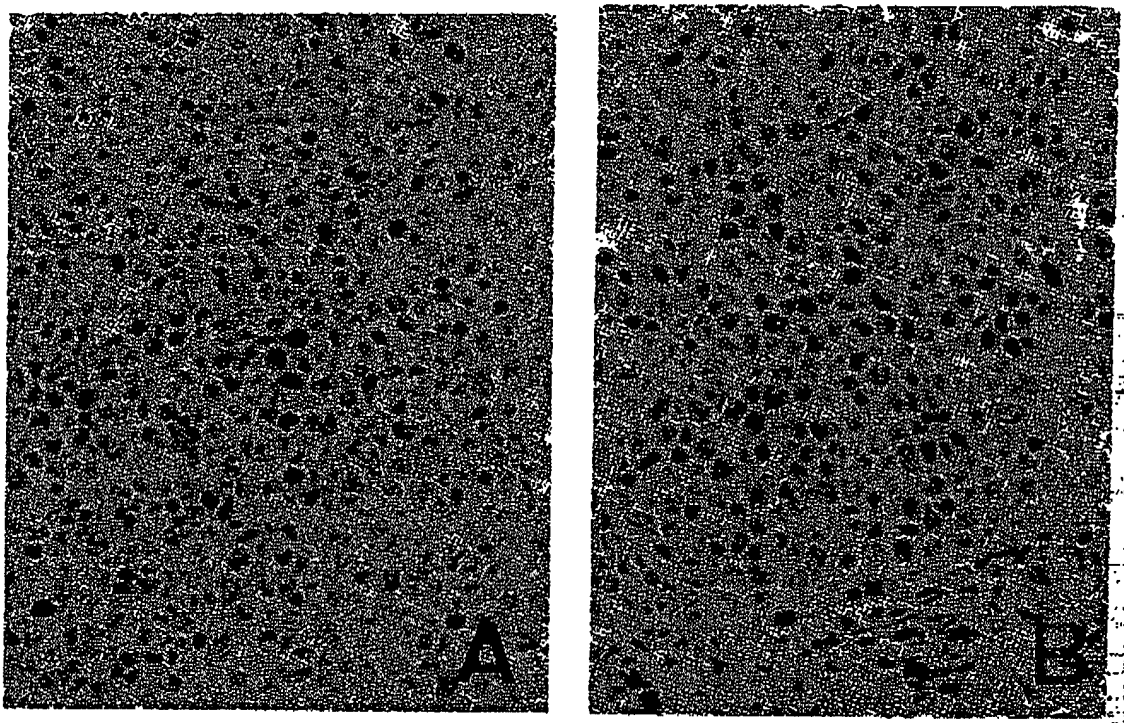

FIG. 4. Lymph node (A) and spleen (B) of a NFS mouse with the mixed leukemia of lymphoblastic and erythroblastic cells (mouse number A121c, in Table 2). A newborn NFS mouse was inoculated with a recombinant Mol-FrMCF (LTR) virus, which induced the leukemia after 60 days. A diffuse proliferation of lymphoblastic leukemic cells with vesicular nuclei are observed in the lymph node. Macrophages ingested many degenerated cells are scattered. In the spleen, a diffuse proliferation of erythroblastic leukemic cells with hyperchromatic nuclei and amphophilic cytoplasm are observed in the red pulp. Smaller nucleated erythrocytes are intermingled. Hematoxylin and eosin. ×500.

as Mol-FrMCF (LTR) virus, but did not induce any mixed leukemia which was induced with Mol-FrMCF (LTR).

Mol-FrMCF (env) virus induced lymphomas in 12 mice and mixed leukemias in 2 mice from a total of 105 mice injected. The mixed leukemias induced with Mol-FrMCF (env) were histologically quite similar to the mixed leukemia induced with Mol-FrMCF (LTR) virus: leukemia of erythroid cells in the spleen and lymphoid cells in lymph nodes.

Replication of the Recombinant Viruses in the Leukemic Tissue

We examined the replication of the recombinant viruses in the leukemic spleens and asked whether any other kinds of virus emerged in the leukemic mice inoculated with the recombinant viruses. It is well known that the MCF virus emerges in the leukemic mice when leukemias are induced with ecotropic Friend or Moloney viruses, although the role of the emerged MCF virus on the leukemogenesis is still unknown (Troxler *et al.*, 1980; Ishimoto *et al.*, 1981).

The presence of various phenotypes of leukemia viruses in the enlarged spleens of mice inoculated with parental and recombinant viruses was examined (Table 2). We tried to detect whether a dualtropic MCF virus or a xenotropic virus emerged in the leukemic tissue inoculated with ecotropic Mol-FrMCF (LTR) or FrMCF-Mol (env), and whether the XC-positive ecotropic virus emerges in the leukemic tissue inoculated with the dualtropic FrMCF-Mol (LTR) and Mol-FrMCF (env).

TABLE 2
PHENOTYPES OF LEUKEMIA VIRUSES DETECTED IN THE ENLARGED SPLEENS OF MICE INOCULATED WITH PARENTAL AND RECOMBINANT VIRUSES

Virus inoculated	5% Crude homogenate of spleen from leukemic mouse: Lymphoid (L), erythroid (E), or mixed leukemia (E + L) (mouse number)	Titer (log PFU or FFU/0.2 ml) of phenotype (symbol or phenotype)[a]: E(e)[b]	E(x)[c]	X(x)[d]	X(e)[e]	D(x)[f]	D(e)[g]
Parental virus							

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S mouse was inoculated
after 60 days. A diffuse
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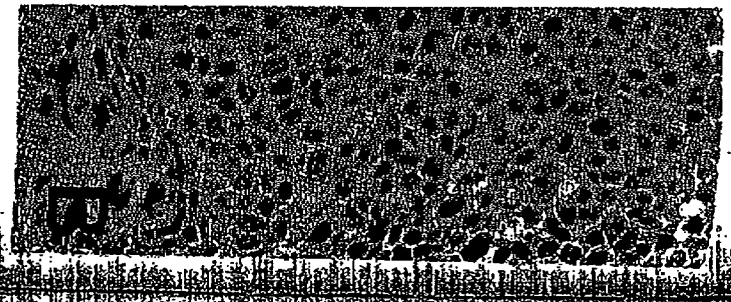

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PHENOTYPES OF LEUKEMIA VIRUSES DETECTED IN THE ENCAPSULED SPLEENS OF MICE INOCULATED WITH PARENTAL AND RECOMBINANT VIRUSES

Virus inoculated	Lymphoid (L), erythroid (E), or mixed leukemia (E + L) (mouse number)	Titer (log PFU or FFU/0.2 ml) of phenotype (symbol or phenotype)[a]					
		E(e)[b]	E(x)[c]	X(x)[d]	X(e)[e]	D(x)[f]	D(e)[g]
	5% Crude homogenate of spleen from leukemic mouse						
Parental virus							
Friend-MCF	E	N[h]	—	N	N	1.0	1.6
	E	N	—	0.8	0.5	2.3	2.0
Ecotropic moloney	L	5.6	—	1.0	0.8	N	1.6
	L	5.8	—	N	0.6	0.3	1.0
Recombinant virus							
FrMCF-Mol (LTR)	L	N	—	0.5	—	0.5	—
	L	N	—	1.0	—	0.5	—
	L	N	—	1.3	—	1.6	—
Mol-FrMCF (LTR)	E	4.7	—	N	0.5	N	1.0
	E	4.8	—	N	0.3	N	1.5
	E	5.0	—	N	1.0	N	1.3
	E + L (A129c)	5.0	—	3.0	4.3	4.0	4.3
	E + L	5.6	—	N	0.6	N	1.6
	E + L (A121c)	5.3	—	2.6	3.0	3.0	3.3
FrMCF-Mol (env)	E	4.9	N	N	N	N	N
	E	5.3	N	N	0.3	0.8	0.8
	E	5.8	N	N	0.8	0.5	1.3
Mol-FrMCF (env)	L	N	N	2.3	—	3.8	—
	L	N	N	1.4	—	3.0	—
	E + L	N	N	1.6	—	2.6	—

[a] The actual viruses detected for each phenotype are very complicated. For example, phenotype X(e) includes at least xenotropic and MCF viral genomes with an ecotropic and dualtropic coat; phenotype X(x) includes xenotropic and MCF viral genomes with a xenotropic or dualtropic viral coat.

[b] Ecotropic virus with ecotropic host range, detected by infecting SC-1 cells and uv-XC test (Rowe *et al.*, 1970).

[c] Ecotropic virus with xenotropic host range, detected by infecting mink cells, uv irradiating, and overlaying mink cells with SC-1 cells 3 days postinfection, followed 3 days later by uv-XC test (Ishimoto *et al.*, 1977).

[d] Xenotropic of dualtropic virus with xenotropic host range, detected by infecting mink S + L− cells and counting transformed foci (Ishimoto *et al.*, 1977).

[e] Xenotropic of dualtropic virus with ecotropic host range, detected by infecting SC-1 cells, 3 days later uv-irradiating and overlaying them with mink S + L− cells, and counting transformed foci (Ishimoto *et al.*, 1977).

[f] Dualtropic MCF virus with xenotropic host range, detected by infecting mink cells and counting mink cell foci.

[g] Dualtropic MCF virus with ecotropic host range, detected by infecting SC-1 cells, 3 days later uv-irradiating and overlaying them with mink cells, and counting mink cell foci.

[h] Not detected.

viruses. In general, when dualtropic MCF viruses were inoculated, the titer of the virus detected in leukemic spleen was very low, and XC-positive viruses were not detected in it. However, when ectropic viruses were inoculated, the virus replicated very well in the leukemic spleen and dualtropic MCF viruses were often detected in the leukemic spleens. The emerged dualtropic MCF virus was usually detected as a phenotypically mixed virus: dualtropic MCF virus genome with ecotropic viral coat. Strikingly, the titer of the emerged MCF virus in two of the three lymphoid leukemic mice induced with Mol-FrMCF (LTR) was abnormally high (mouse number A129c and A121c, in Table 2).

DISCUSSION

Several recent observations indicate that LTR sequences determine the leukemogenicity and tissue specificity of MuLV by conferring tropism for the target cells (Chatis *et al.*, 1983; DesGroseille *et al.*, 1983; Lenz *et al.*, 1984).

Chatis *et al.* (1982) showed with data obtained from an *in vitro* recombinant virus constructed between ecotropic Friend and Moloney viruses that the lymphoid nature of leukemia induced by the Moloney virus is probably determined by the LTR sequences. Lymphoid pathogenesis of our recombinant viruses. FrMCF-Mol (LTR) and Mol-FrMCF (env), constructed between Friend-MCF and Moloney viruses also confirmed it.

Our data presented here showed that the erythroid nature of leukemia is mainly determined by the LTR sequence. Mol-FrMCF (LTR) virus was derived primarily from the Moloney virus but had 596 nucleotides of Friend-MCF virus information at its 3′ end. FrMCF-Mol (env) virus was derived from Friend-MCF virus except for 2.7-kbp nucleotides of Moloney virus information including most of *env* gene. The erythroid pathogenicity of Mol-FrMCF (LTR) and FrMCF-Mol (env) suggests that the target cell specificity of the erythroid leukemia virus resides primarily in the U3 region of LTR.

The ultimate mechanism of tumorigenesis by murine leukemia virus may involve the activation of specific cellular genes by insertion of the viral genome into cellular sequences. Although we do not know how the LTR determined the disease specificity of Friend-MCF and Moloney virus, it can be expected that some sequence in LTR such as promotor and enhancer sequences are involved through the activation of some cellular genes. Revealing the DNA sequence of Friend-MCF virus LTR through its comparison with Moloney virus, has not enabled the identification of the sequence responsible for the erythroid or lymphoid leukemia induction due to the large number of differences in enhancer and promoter sequences between them (Adachi *et al.*, 1984). Recently some recombinants have been constructed in our laboratory whose genomes were derived primarily from Friend-MCF virus, but possessing enhancer or promotor sequences of Moloney virus. These might reveal the possible role of the enhancer and promotor sequences on determining the disease specificity.

Several investigators have suggested that the viral *env* gene might play an important role in leukemogenesis (Linemeyer *et al.*, 1982; Oliff and Ruscetti, 1983). Comparison of the nucleic and amino acid sequences of Friend-MCF virus with sequences of ecotropic Friend virus showed the *env* gene region of Friend-MCF virus to be substituted with some endogenous nonecotropic virus like sequences in the N-terminal portion of envelope protein (Adachi *et al.*, 1984). The substituted envelope protein may be responsible for the erythroid leukemia induction, since the nucleotide sequences of the *env* gene of Friend-MCF virus and the *env*-related sequence of Friend-SFFV were quite homologous. The present study investigated whether the substituted *env* gene sequence in Friend-MCF virus was responsible for the induction of erythroid leukemia. We had expected that Friend-MCF virus could not be converted to lymphoma-inducing virus by exchanging the LTR whereas ecotropic Friend virus would be. The data suggest the disease specificity of the

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velopment of mixed leukemia with Mol-
MCF (LTR) and Mol-FrMCF (env) vi-
es in some mice still suggests that the
get cell specificity of erythroid and
ymphoid leukemia may reside partially
the *env* or the *pol-env* gene region.
the 621- and 596-nucleotide *Ban*III-*Kpn*I
gments from Moloney and Friend-MCF
rus, respectively, encompass the se-
uences coding for the short part of the
-terminal of p15E in addition to the 5′
de of LTR. But the possible influence of
he short sequence coding the p15E portion
of *env* on disease specificity remains un-
certain. Further clarification of these
problems might be facilitated by con-
structing recombinant viruses in which
the LTR and *env* gene are exchanged
between a nononcogenic virus and Friend-
MCF or Moloney virus at the two restric-
tion endonuclease sites located in the LTR.

Leukemogenesis by murine leukemia
viruses is a multigene phenomenon, being
determined by host and viral genetic fac-
tors. Our experiments showed that the
incidences of leukemia induced with eco-
tropic viruses were much higher than
those of leukemia induced with dualtropic
MCF viruses, even if the disease specificity
of the dualtropic MCF virus was the same
as that of the ecotropic virus possessing
the same LTR. The low viral growth of
the dualtropic MCF virus in mice may be
an important factor for the low incidence
of leukemia in mice inoculated with dual-
tropic MCF virus. The titers of the MCF
virus detected in the leukemic spleen of
mice were usually much lower than the
titers of MCF virus detected in the super-
natant of SC-1 or mink cells *in vitro*: the
former were usually one-hundredth of the
latter or less (unpublished data). However,
the titer of the ecotropic virus such as
Friend virus in the supernatant of SC-1
cells *in vitro* and the titer of the same
virus detected in the leukemic spleen as
5% homogenate were almost the same.

Bosselman *et al.* (1982) showed that the
information for the dualtropic host range
and cytopathic effect of Moloney-MCF vi-
rus lies in the 3′ half of Moloney-MCF
DNA which encompasses the 3′ half of
the *pol* gene, the complete *env* gene, and
the LTR. Our recombinant virus showed
that the information for the cytopathic
effect of Friend-MCF virus lies outside
the LTR. However, the relationship be-
tween the leukemogenicity and mink cell
foci-inducibility by the MCF viruses is
still unknown.

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H, ^{13}C, and ^{31}P

Virus: Detection

R. VIRUDACH

*Purdue Universit

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Cowpea mosaic v icosahedral partic the comovirus gro divided genome. T bonucleic acids w 1.2×10^6 and 1.9 separately in ident is composed of 6 different proteins 22,000 and 42,000. lating CpMV from yields a mixture o and filled particle smaller RNA and The particles, whi a cesium chloride g

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that the −343 fragment and a−941 fragment have no significant difference in promoter activity (data not shown).

Regenerated transformed tobacco plants contain one to two copies per genome of the NOS promoter-*npt*-II and 35S promoter-*hgh* genes, determined by Southern blots (data not shown). This low copy number in plants, compared with the high copy numbers in calli (Fig. 2), could be explained by the transformation procedure (co-cultivation versus wounding) or by the type of T-DNA transfer event involved (short verus long). Northern blots show that leaves of plants transformed with each of the four 35S promoter deletions contain the same 2.3-kb *hgh* RNA found in transformed calli (Fig. 3*c*). By S_1 analysis the 5′ end of this leaf *hgh* RNA was found to be identical to that of the callus *hgh* RNA (data not shown). The effects of the deletions on promoter activity in transformed plant leaves (Fig. 3*c*) closely resemble results described for the transformed calli. The 35S promoter was also active in roots, petals and stems of transformed plants (Fig. 3*c*), with deletions having no specific effects on tissue expression (data not shown). The ratio of *hgh*/*npt*-II transcripts is constant in the different tissues. Both transcripts appear reduced in the root RNA preparation, but this could be due to varying amounts of ribosomal RNA contamination in the polyadenylated RNA preparations.

Here, we have shown that although the normal host range of CaMV is limited to members of the Cruciferae, the 35S promoter is active when isolated as a fragment from the viral genome and integrated into the tobacco genome. Thus, accurate transcription from the 35S promoter does not depend on any *trans*-acting viral gene products. The ability to regenerate tobacco plants from transformed protoplasts has allowed us to demonstrate that the 35S promoter is expressed in leaves, stems, roots and petals.

Promoter deletion analysis in transformed calli and plants showed that a −46 fragment, which does contain a TATA-box sequence (see Fig. 1), produces a low level of correctly initiated transcripts. The region between −46 and −105 which greatly increases the level of transcription contains a CAAT-box sequence, an inverted repeat region and a sequence resembling the consensus core for enhancers in animal systems ($GTGG^{AAA}_{TTT}G$) (ref. 10; see Fig. 1). We are investigating whether one or more of these features plays a substantial role in increasing the level of 35S promoter activity or could act to increase transcription from a foreign promoter.

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Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells

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Epstein–Barr virus (EBV) infects human B lymphocytes, transforming the infected cells into dividing blasts that can proliferate indefinitely (see ref. 1 for a review). The viral genome of 172 kilobase pairs (kbp) is a plasmid in most transformed cells[2–4]. We have identified a region of EBV DNA, termed *oriP* (nucleotides 7,333–9,109 of strain B95–8), which acts in *cis* to permit linked DNAs to replicate as plasmids in cells containing EBV DNA[5]. We have postulated the existence of a *trans*-acting gene allowing *oriP* function. Here we report that this gene lies in a 2.6-kbp region of the viral genome (nucleotides 107, 567–110, 176) which encodes the EBNA-1 antigen[6–8]. We show that circular DNAs containing *oriP*, the EBNA-1 gene and a selectable marker replicate autonomously in cells derived from at least four developmental lineages and from at least three species. We also find that the one-third of the *EBNA-1* gene repetitive in sequence is not essential for the *trans*-acting function that EBNA-1 gives *oriP*.

To map the gene encoding the proposed *trans*-acting function, overlapping segments of the EBV genome were first introduced individually into the human thymidine kinase (TK)-negative cell line 143, using a set of recombinant plasmids selected using the antibiotic G418 (ref. 5). These G418-resistant cell lines were transfected subsequently with the hypoxanthine-aminopterin thymidine (HAT)-selectable plasmid pΔ*π*TK or its derivative, pTKBamC, which contains *oriP*. Only those G418-resistant cells that carried EBV DNA mapping from *Bam*HI-Z to *Sal*I-F (Fig. 1*a*, *b*) supported pTKBamC as a plasmid. Three of four 143 clones carrying pBamZRSalF (termed 143/BamZRSalF) could be transfected stably 5–30-fold more efficiently with the *oriP*-containing plasmid, pTKBamC, than with its parent, pΔ*π*TK (Table 1, experiment 1). Analysis of DNA from the HAT-resistant clones showed that the 143/BamZRSalF cell lines efficiently transfected by pTKBamC contained it as a plasmid at two to four copies per cell (Table 1). 143 clones carrying all other regions of the EBV genome showed no increase in transfection frequency-dependent on *oriP* and did not contain pTKBamC as a plasmid. These results imply that the EBV DNA spanning the *Bam*HI-Z/*Sal*I-F fragment encodes the proposed *trans*-acting product which allows maintenance of *oriP*-bearing plasmids.

The only viral product encoded in this region of the genome and known to be expressed in EBV-transformed cells is the nuclear antigen EBNA-1. It is encoded in the *Bam*HI-K fragment, which lies within the *Sal*I-F fragment of EBV DNA[6–8]. A 2.9-kbp *Bam*HI/*Hin*dIII subfragment of *Bam*HI-K encodes most, if not all, of the EBNA-1 polypeptide[9,10] (see maps of Fig. 1). This 2.9-kbp subfragment was cloned into a G418-selectable plasmid containing the transcriptional enhancer of simian virus 40 (SV40), and the resulting plasmid, pSVoB-H2.9, was introduced into 143 cells. Two of five such cell lines, 143/SVoB-H2.9 clones 1 and 4, expressed levels of EBNA-1 that could be detected by anti-complement immunofluorescence (our unpublished observations). When we transfected pTKBamC into these two 143-derived clones, we efficiently selected HAT-resistant colonies in which one to three copies of the *oriP*-bearing plasmid were maintained per cell (Table 1). Thus, the *trans*-acting product required for *oriP* function maps in the 2.9-kbp region encoding EBNA-1. The SV40 enhancer was required for efficient expression of the *trans*-acting function from the integrated 2.9-kbp fragment (data not shown). This finding is consistent with the observation that the mRNA for this region is a 3.7-kb transcript beginning upstream of *Bam*HI-K[11,12].

Table 1 Abilit

Cell line	
Expt 1	
143/BamZR	
Clone 1	
Clone 2	
Clone 3	
Clone 4	
All others	
Expt 2	
143/BamZR	
Clone 2	
143/SVoB-H	
Clone 1	
Clone 4	
Expt 3	
143/SVoB-H	
Clone 4	
143 control	

Clonal, G41
copies of the
described[5]. Co
143/BamZRS
the EBV DNA
clones carryin
EBV genome[5]
tested. pSVo
*Bam*HI/*Hind*
site and the *H*
containing *H*
the *Hin*dIII si
and Van der
2.5 μg of plas
HAT or 150 μ
vector pΔ*π*T
construction
oriP-containi
to the drug h
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143/BamZRS
not to be resi
HAT-resistan
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* Although
than 10-fold
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8-resistant
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Table 1 Ability of 143 cells carrying integrated fragments of EBV DNA to maintain plasmids containing *oriP*

Cell line	No. of colonies after transfection with: pΔπTK	pTKBamC	Plasmid molecules per cell: pΔπTK	pTKBamC
Expt 1				
143/BamZRSalF				
Clone 1	48	800	0	2
Clone 2	92	480	0	4
Clone 3	24	36	0	0
Clone 4	18	540	ND	ND
All others	18–190*	8–80*	ND	0
Expt 2				
143/BamZRSalF				
Clone 2	3	410	ND	ND
143/SVoB-H2.9				
Clone 1	2	450	0	1.5
Clone 4	3	650	0	3
	pHyg	pHEBo2	pHyg	pHEBo2
Expt 3				
143/SVoB-H2.9				
Clone 4	3	6,000†	ND	3
143 control	5	15	ND	ND

Clonal, G418-resistant derivatives of 143 cells carrying integrated copies of the indicated plasmid were constructed as previously described[5]. Cell lines are named for the plasmid carried; for example, 143/BamZRSalF carries pBamZRSalF (ref. 5). pBamZRSalF contains the EBV DNA shown in Fig. 1*a*, 1*b*. 'All others' refers to different 143 clones carrying each of nine plasmids that span the remainder of the EBV genome[5]. Four clonal lines carrying each integrated plasmid were tested. pSVoB-H2.9 was constructed by inserting the 2.9-kpb *Bam*HI/*Hin*dIII fragment of *Bam*HI K (Fig. 1) between the *Bam*HI site and the *Hin*dIII site of pKan2 (ref. 5) and by inserting the origin-containing *Hpa*II/*Hin*dIII fragment of SV40 between the *Cla*I site and the *Hin*dIII site of the resulting plasmid. Using the method of Graham and Van der Eb[20], ~1×10^6 cells from each line were transfected with 2.5 μg of plasmid DNA and selected after 24 h in medium containing HAT or 150 μg ml^{-1} hygromycin B (Calbiochem). The HAT-selectable vector pΔπTK carries the *tk* gene of herpes simplex type 1 (see the construction of pKan2 in ref. 5). pTKBamC is pΔπTK carrying the *oriP*-containing *Bam*HI-C fragment of EBV. pHEBo2 confers resistance to the drug hygromycin B[13] and carries *oriP* (ref. 14 and Fig. 1); pHyg is identical to pHEBo2 except that it lacks the EBV sequences. Selected colonies were amplified and analysed for the presence of plasmid molecules as shown in Fig. 2. The limit of detection was 0.2 copies per cell. In addition, pTKBamC was recovered from DNA of transfected 143/BamZRSalF clones 1 and 2 by transformation of *E. coli* and shown not to be rearranged, as described previously[5]. For 'all others', a single HAT-resistant clone from one line carrying each region of EBV DNA was analysed. Using a probe specific for the *oriP* region, each of these was shown to carry integrated copies of pTKBamC. ND, not determined.

* Although the transfection efficiency of these lines varied by more than 10-fold, the ratio of the number of colonies obtained with pTKBamC to the number obtained with pΔπTK was between 0.3 and 0.8 for every cell line.

† The fraction of 143/SVoB-H2.9 clone 4 cells that can be stably transfected with pHEBo2 is 0.5–1%, close to the 1–2% that can be transfected with SV40 DNA to express T antigen transiently.

We constructed plasmids carrying *oriP*, the *EBNA-1* gene and a selectable marker and found that they replicated autonomously in a variety of cultured cells. Because many cell lines are resistant to high concentrations of G418, we first made the *oriP*-bearing vector pHEBo2, which confers resistance to the drug hygromycin B[13,14] (Fig. 1). pHEBo2 was >100-fold more efficient in conferring drug resistance to 143 cells expressing EBNA-1 than to the same number of control cells (Table 1, experiment 3). A similar effect of the *EBNA-1* gene was observed when it was inserted in pHEBo2. Five different derivatives of pHEBo2 carrying the *EBNA-1* gene yielded hygromycin B-resistant colonies 10–100 times more efficiently than did pHEBo2 when transfected into HeLa cells; all five were maintained as plasmids in the resistant cells (Fig. 1*b*).

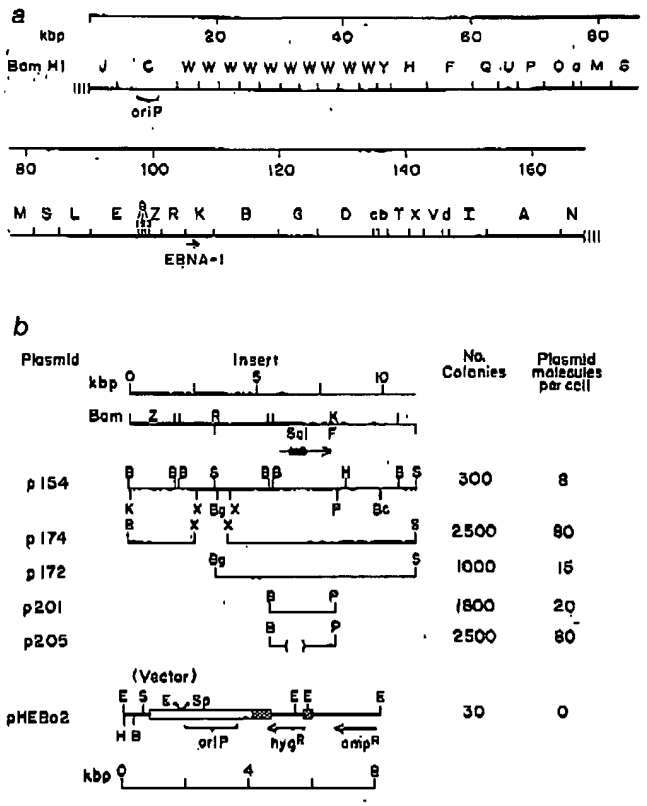

Fig. 1 *a*, Map of linear, Epstein-Barr viral DNA indicating all but the smallest *Bam*HI restriction endonuclease fragments[10] and the positions of *oriP* and the *EBNA-1* gene. The vertical lines represent the terminal repetitions found in the viron DNA. *b*, Structure and function of plasmids carrying *oriP* and the *EBNA-1* gene. The vector pHEBo2 is shown at the bottom, linearized at the *Eco*RI site derived from pBR322. Its construction is described elsewhere[14]. The bacterial gene for hygromycin B resistance[13] is expressed using the promoter and polyadenylation signals of the herpes simplex *tk* gene (hatched boxes). *OriP* is present on an *Sst*I/*Sst*II fragment of EBV DNA[5] (open box). The relevant EBV *Bam*HI and *Sal*I fragments are shown at the top. The arrow represents the open reading frame BKRF1[10] (*EBNA-1*), with the thickened part representing the triplet repeats. In the middle of the figure, plasmids are represented by the DNA segment inserted into pHEBo2. Inserts of the first three plasmids are between the *Bam*HI and *Sal*I sites of pHEBo2. For p201 and p205, the DNA was inserted by blunt-edged ligation between *Hin*dIII and *Sph*I sites. The orientations of all inserts relative to the vector are as shown. p174 was derived from p154 by deletion of the DNA between the *Xho*I sites shown. The insert of p205 carries a 700 ± 20-bp deletion at the site of the triplet repeats. The triplet repeat array is 717 bp long in the B95-8 strain[10] from which the *EBNA-1* gene was cloned. The deletion was obtained by subcloning from the Ch4A clone EB 62-71 in which this region spontaneously deletes[15]. We have not determined the sequence at the boundaries of the deletion, but note that deletion of the triplet repeats by homologous recombination would preserve the reading frame. A similarly obtained deletion was reported to be in frame[9]. All restriction enzyme cleavage sites relevant to this report are indicated for the insert of p154. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. Methods. Plasmids were tested by transfecting 1×10^6 HeLa cells as described in Table 1 legend and selecting for resistance to hygromycin B at 150 μg ml^{-1}. Resistant colonies were counted after 11–21 days of selection, trypsinized and carried or expanded for 18–23 population doublings. The amount of free plasmid present in the cell populations was determined as shown in Fig. 2*a*, except that DNAs were usually not digested with endonucleases. The average of determinations from at least two independent transfections is shown.

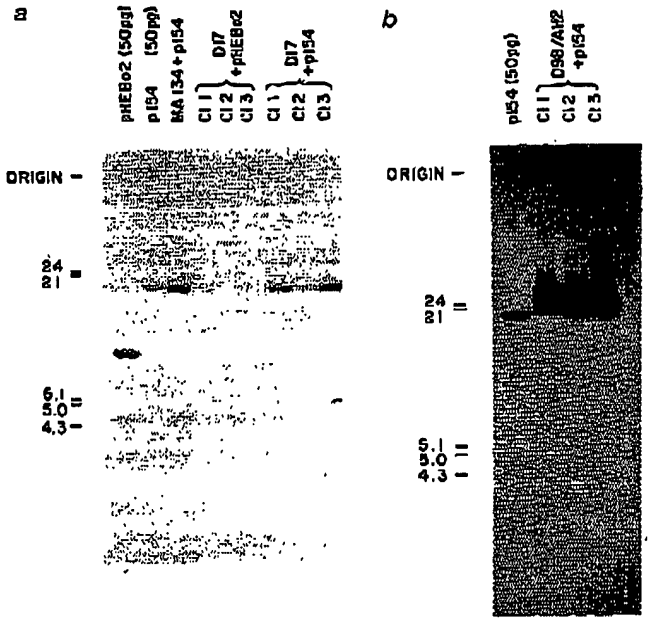

Fig. 2 Analysis of EBV-derived plasmids in transfected cells. DNAs were separated by electrophoresis in 0.5% agarose and detected by the method of Southern[27] using the plasmid pHyg as probe, labelled isotopically to a specific activity of 2–4 × 10^8 c.p.m. μg^{-1}. *a*, DNA was isolated from supernatants of Hirt extracts[28] of 2×10^6 hygromycin B-resistant MA134 cells (a pool of 34 clones) or D17 cells (individual clones) transfected with the indicated plasmids. DNAs were linearized by cleavage with the following enzymes: MA134 or D17 plus p154 with *Bcl*I, D17 plus pHEBo2 with *Hin*dIII, pHEBo2 standard with *Hin*dIII, and p154 standard with *Kpn*I. Cleavage of p154 DNA isolated from the MA134 and D17 cells by *Bcl*I shows that the plasmid had lost its methylation, and hence, had replicated. *b*, Total DNA from 1×10^6 cells of three D98/AH2 clones carrying p154 was analysed after digestion with *Bgl*II. A long exposure of the autoradiogram (96 h) is shown to demonstrate the absence of any additional bands which could result were vector sequences integrated. This experiment does not eliminate the possibility that these lines could carry integrated fragments of p154 which lack homology to pHyg. The positions and sizes in kbp of DNA size markers, λ DNA cut with *Eco*RI and *Hin*dIII, are indicated.

Table 2 Ability of EBV-derived replicons to function in cells of various origins

Cell line	Plasmid	Frequency of stable transfection	Plasmid molecules per cell
Human			
D98/AH2	p154	1×10^{-4}	7,12,14
143	p172	2×10^{-4}	1,10
143	pHEBo2	5×10^{-5}	0
293	p154	1×10^{-4}	35
293	pHEBo2	2×10^{-5}	0
Wilson	p174	3×10^{-6}	10, 16
Wilson	pHEBo2	$<2 \times 10^{-7}$	—
K562	p172	2×10^{-5}	9,27,90
Monkey			
MA134	p154	3×10^{-5}	5
MA134	pHEBo2	$<5 \times 10^{-7}$	—
Dog			
D17	p154	6×10^{-4}	1.5,5,5
D17	pHEBo2	2×10^{-4}	0
Rodent			
BALB/c3T3	p174	1×10^{-4}	0*
BALB/c3T3	pHEBo2	1×10^{-4}	0
HTC-A	p172	9×10^{-4}	0
V79	p174	9×10^{-4}	0, 0, 0*

Plasmid DNAs (Fig. 1*b*) were introduced by the calcium phosphate method for all cell lines except K562 and Wilson, for which fusion with *E. coli* spheroplasts[21] and electroporation[22], respectively, were used as described elsewhere[14]. Transfected cells were selected with the following concentrations of hygromycin B (μg ml^{-1}): D98/AH2 (ref. 23), 143 (ref. 24), 293 (ref. 25), BALB/c3T3 and MA134, 150; D17 (obtained from American Type Culture Collection), 200; Wilson (obtained from Dr Ian McGrath), K562 (ref. 26) and V79, 300; HTC-A (obtained from Dr H. Pitot), 500. < indicates that no resistant clones appeared within the number of cells tested. No resistant colonies arose from any cell line without transfection. To determine the number of plasmid molecules per cell, individual colonies or pools of colonies were amplified and their DNAs analysed as in Fig. 2, using pHEBo2 DNA as a probe. Limits of detection were ≈0.2 copies per cell. Where pools of colonies were analysed, a single value is given. Where individual colonies were analysed, the value obtained for each clone is given.

* These resistant colonies, obtained from the rodent cell lines, were shown to carry integrated vector sequences. In addition, BALB/c3T3 cells carrying integrated pSVoB-H2.9 and expressing levels of EBNA-1 detectable by anti-complement immunofluorescence could not maintain the *oriP*-bearing plasmid pHEBo2 (limit of detection 0.06 copies per cell).

That the smallest of these plasmids, p201 and p205, work efficiently implies that the *trans*-acting function required by *oriP* maps entirely in a 2.6-kbp *Bam*HI/*Pvu*II fragment and does not include all of the *EBNA-1* coding sequences (Fig. 1*b*). Between the *Bam*HI site and the *Pvu*II site lies an open reading frame of the correct size to encode the EBNA-1 polypeptide[10]. About one-third of this open reading frame is a repetitive sequence of glycine and alanine codons which encodes part of EBNA-1[7,8] (arrow in Fig. 1*b*). A spontaneous deletion of the repetitive region of the *EBNA-1* gene was obtained from a recombinant bacteriophage clone grown in *recA*$^+$ *Escherichia coli*[15]. If deletion of the triplet repeats occurred by homologous recombination, the reading frame would be preserved. A similar, spontaneous deletion was observed by Fischer *et al.* to be in-frame[9]. We found that p205, which carries this deletion, replicates more efficiently than p201 (Fig. 1*b*), indicating that most or all of the triplet repeats are dispensable for the *trans*-acting function that the *EBNA-1* gene product gives *oriP*.

These experiments do not imply that transcription of the *EBNA-1* gene on any of the described plasmids occurs as it does from the viral genome. If the *EBNA*-1 gene is placed on pHEBo2 in the opposite orientation to that shown in Fig. 1*b*, it fails to function unless a transcriptional enhancer is provided (data not shown). The expression of the *trans*-acting function obtained with plasmids such as p201 may depend on a promoter(s) present on pHEBo2, perhaps one of those found near the bacterial ampicillin resistance gene[16,17].

EBV-derived replicons, plasmids carrying both *oriP* and the *EBNA-1* gene, have been introduced into a variety of cell lines (Table 2). Figure 2 shows the ability of these plasmids to replicate in human D98/AH2 cells (of epithelial origin), MA134 African green monkey kidney cells and D17 dog fibrosarcoma cells. pHEBo2, which lacks the *EBNA-1* gene, was not found as a plasmid in the D17 cells (Fig. 2*a*). Plasmid p154, which is pHEBo2 plus the *EBNA-1* gene on an 11-kbp insert, was present as a plasmid at about five copies per cell in a pool of hygromycin B-resistant clones of MA134, at 1.5–5 copies per cell in three clones of D17, and at 7–14 copies per cell in three clones of D98/AH2. Cell clones carrying p154 as a plasmid generally do not have vector sequences integrated into the host chromosomes (Fig. 2*b*). In addition to functioning in human cells of epithelial and fibroblast origin, EBV-derived plasmids were found to be maintained in Wilson cells, an EBV-negative, human B-lymphoma cell line, and in K562 cells, a human erythroleukaemia cell line, but not in three cell lines from rodents (Table 2).

The rate of loss of the EBV-derived replicon, p154, in populations of D98/AH2 grown in the absence of selection was determined using a clonal assay described previously[5]. In the three clones carrying p154 analysed in Fig. 2*b*, the plasmid was lost at rates of 2, 3 and 5% per generation (measured at 18 and 27 generations after removal of the drug). The loss of hygromycin resistance in dividing cells in these three populations is consistent with the absence of integrated copies of the plasmid (Fig. 2*b*). This rate of loss is the same as that for *oriP*-bearing plasmids in cells containing EBV DNA[5]. Thus, the *EBNA-1* gene permits the maintenance of *oriP*-bearing plasmids as efficiently as does all of EBV DNA.

The wide host range and maintenance of the EBV-derived plasmids is in marked contrast to the replication of plasmids derived from BPV and SV40; BPV-derived plasmids have not been reported to replicate in cell lines derived from species other than mouse or rat, and those derived from SV40 are not usually maintained as plasmids[18]. For the human, monkey and dog cell lines mentioned here, we have not observed integration of plasmids carrying *oriP* and a functional *EBNA-1* gene. There is no reason to believe that such plasmids cannot be integrated into host chromosomes, however, as the entire EBV genome has been found integrated in more than one instance[19]. Our results indicate that EBV-derived replicons are more often maintained as plasmids than as integrated molecules in the non-rodent cells we have studied. That EBV-derived plasmids can be maintained in a variety of mammalian cells makes them useful for introducing genes into these cells and for isolating molecular clones of genes from these cells. In particular, plasmids containing both *oriP* and the *EBNA-1* gene should be essential vehicles for identifying and analysing those viral genes that, in addition to *oriP* and *EBNA*-1, are needed to transform human B lymphocytes.

We thank Linda Gritz and Julian Davies for providing us with their plasmid carrying the *hph* (hygromycin B-resistance) gene before its publication, Bart Barrell and co-workers for providing EBV DNA sequences before publication and David Reisman for a clone of the *EBNA-1* gene with the deletion of the triplet repeats. This work was supported by USPHS grants CA-22443, CA-97175 and 5 T32 CA-09075. B.S. was supported by Faculty Research Award 023 from the American Cancer Society.

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Gene deletion and restriction fragment length polymorphisms at the human ornithine transcarbamylase locus

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Deficiency of ornithine transcarbamylase (OTC; EC 2.1.3.3), a hepatic mitochondrial enzyme involved in the detoxification of ammonia[1,2], is a severe inborn error of metabolism. It is an X-linked disorder[3,4] which results characteristically in ammonia intoxication, protein intolerance and mental retardation. Early death of affected hemizygous male infants is common, while clinical manifestations in heterozygous females are variable due to random X-chromosome inactivation[3–5]. Prenatal diagnosis by amniocentesis has not been feasible because OTC is not expressed in amniocytes and because no unusual metabolites can be detected in amniotic fluid. Fetal liver biopsy has been performed for some families at risk[6], but the dangers inherent in this procedure severely limit its usefulness. In this report, we describe the use of a nearly full-length cloned human cDNA[7] to begin to characterize normal and mutant human OTC genes. One of 15 affected males was found to have a partial deletion of the OTC gene. Two distinct restriction fragment length polymorphisms (RFLPs) were identified at the OTC locus using the restriction endonuclease *Msp*I; 69% of women tested were heterozygous for one or both polymorphisms. Identification of these common polymorphisms makes it possible to offer prenatal diagnosis to a large fraction of obligate carriers and to provide information on carrier status to some females at risk.

In an initial experiment, high relative molecular mass (M_r) DNA from two control females was digested independently with restriction endonucleases *Bgl*I, *Eco*RI, *Sst*I, and *Bam*HI and analysed by DNA blot hybridization using the OTC cDNA as probe. No differences between the two controls were detected using these four enzymes. The total size of the gene, estimated by addition of the fragment lengths obtained with any one enzyme, is at least 25–30 kilobases (kb). In a further experiment, the hybridization intensities of all bands showed a clear X-chromosome dosage effect when DNAs from cell lines containing one, two, or four X chromosomes were analysed, indicating that all of the hybridizing fragments are localized to the X chromosome (not shown). The possibility that there are OTC pseudogenes on the X chromosome has not been eliminated.

Figure 1 depicts the hybridization patterns following *Hin*dIII digestion of DNA from seven OTC-deficient males. The pattern from the DNA of six of the patients is indistinguishable from that of controls, whereas the pattern from the DNA of one patient (lane 6) does not include the 3.1-kb band. Digestion of this patient's DNA with five other restriction endonucleases indicated that one hybridizing band was absent with each enzyme; no new bands were detected (not shown). This partial deletion has been localized to the 3′ portion of the gene, based on hybridization experiments with 3′-fragments of the OTC cDNA (not shown). To date, we have examined DNA from 15 OTC-deficient males and have identified only this one with a gene deletion.

Because a substantial proportion (up to one-third) of cases of an X-linked lethal disorder may be due to new mutations[8], we examined the DNA from the mother and maternal aunt of patient 6 to determine if they were carriers for this deletion. Figure 2*a* shows the middle portion of a blot of *Eco*RI-digested DNA from two control females (lanes 1, 2), the maternal aunt

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Identification and Characterization of Three Novel β1,3-*N*-Acetylglucosaminyltransferases Structurally Related to the β1,3-Galactosyltransferase Family*

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We have isolated three types of cDNAs encoding novel β1,3-*N*-acetylglucosaminyltransferases (designated β3Gn-T2, -T3, and -T4) from human gastric mucosa and the neuroblastoma cell line SK-N-MC. These enzymes are predicted to be type 2 transmembrane proteins of 397, 372, and 378 amino acids, respectively. They share motifs conserved among members of the β1,3-galactosyltransferase family and a β1,3-*N*-acetylglucosaminyltransferase (designated β3Gn-T1), but show no structural similarity to another type of β1,3-*N*-acetylglucosaminyltransferase (iGnT). Each of the enzymes expressed by insect cells as a secreted protein fused to the FLAG peptide showed β1,3-*N*-acetylglucosaminyltransferase activity for type 2 oligosaccharides but not β1,3-galactosyltransferase activity. These enzymes exhibited different substrate specificity. Transfection of Namalwa KJM-1 cells with β3Gn-T2, -T3, or -T4 cDNA led to an increase in poly-*N*-acetyllactosamines recognized by an anti-i-antigen antibody or specific lectins. The expression profiles of these β3Gn-Ts were different among 35 human tissues. β3Gn-T2 was ubiquitously expressed, whereas expression of β3Gn-T3 and -T4 was relatively restricted. β3Gn-T3 was expressed in colon, jejunum, stomach, esophagus, placenta, and trachea. β3Gn-T4 was mainly expressed in brain. These results have revealed that several β1,3-*N*-acetylglucosaminyltransferases form a family with structural similarity to the β1,3-galactosyltransferase family. Considering the differences in substrate specificity and distribution, each β1,3-*N*-acetylglucosaminyltransferase may play different roles.

A family of human β1,3-galactosyltransferases (β3Gal-Ts)[1] consisting of five members (β3Gal-T1, -T2, -T3, -T4, and -T5) was recently identified (1–4). The first β1,3-galactosyltransferase (β3Gal-T1), which catalyzes the formation of type 1 oligosaccharides, was isolated by us using an expression cloning approach (1). Expression patterns of β3Gal-T1 and type 1 oligosaccharides strongly suggested the existence of β3Gal-T1 homologs. For instance, type 1-derived oligosaccharides such as sialyl-Le^a were known to be expressed in colon and pancreatic cancer cell lines, whereas expression of β3Gal-T1 was detected in brain, but not in cancer cells. Our early approach using Southern hybridization failed to detect the existence of β3Gal-T1 homologous genes. However, recent accumulation of nucleotide sequence information on human cDNAs and genes such as expressed sequence tags (ESTs) enabled us to search homologous genes that do not have high similarity as detected by hybridization, but show significant similarity. A homology search based on the nucleotide or amino acid sequence of β3Gal-T1 led to the isolation of β3Gal-T2, -T3, and -T4, indicating that β3Gal-Ts form a family (1–3).

β3Gal-T2 catalyzed a similar reaction, but showed different substrate specificity compared with β3Gal-T1. The activity of β3Gal-T3 has not been detected, whereas the corresponding mouse enzyme exhibits weak β3Gal-T activity for both GlcNAc and GalNAc (5). On the other hand, β3Gal-T4 transfers galactose to the GalNAc residue of asialo-G_{M2} or G_{M2} to catalyze the formation of asialo-G_{M1} or G_{M1}, respectively (3). β3Gal-T4 may be a human homolog of rat G_{M1} and G_{D1} synthases (6) since these enzymes shows 79.4% identity at the amino acid level.

A PCR cloning approach using degenerate primers corresponding to conserved regions in the β3Gal-T family has enabled us to isolate a fifth member (β3Gal-T5) of this family, which catalyzes the synthesis of type 1 oligosaccharides and is the most probable candidate involved in the biosynthesis of a cancer-associated sugar antigen, sialyl-Le^a, in gastrointestinal and pancreatic cancer cells (4).

Very interestingly, a β1,3-*N*-acetylglucosaminyltransferase (designated β3Gn-T1) has been recently isolated based on the structural similarity to the β3Gal-Ts (7). β3Gn-T1 showa sig-

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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB049584 (β3Gn-T2), AB049585 (β3Gn-T3), and AB049586 (β3Gn-T4).

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[1] The abbreviations used are: β3Gal-Ts, UDP-galactose:β-*N*-acetylglucosamine β1,3-galactosyltransferases; EST, expressed sequence tag; G_{M1}, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer; G_{M2}, GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer; G_{D1}, Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glc-Cer; PCR, polymerase chain reaction; β3Gn-T, UDP-GlcNAc:β-galactose β1,3-*N*-acetylglucosaminyltransferase; bp, base pair(s); kb, kilobase pair(s); LEA, *L. esculentum* agglutinin; PWM, pokeweed mitogen; MOPS, 4-morpholinepropanesulfonic acid; LNnT, lacto-*N*-neotetraose; *p*-LNnH, *p*-lacto-*N*-neohexaose; LNT, lacto-*N*-tetraose; LNFP, lacto-*N*-fucosylpentaose; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction.

nificant overall similarity to β3Gal-Ts (15–19%) and shares motifs conserved among the β3Gal-Ts, but is structurally distinct from another type of β1,3-*N*-acetylglucosaminyltransferase (iGnT) that was isolated by expression cloning using an anti-i-antigen antibody (8). β3Gn-T1 exhibits β1,3-*N*-acetylglucosaminyltransferase activity instead of β1,3-galactosyltransferase activity. This result provides an exception that a glycosyltransferase structurally related to the β3Gal-T family uses distinct donor (GlcNAc *versus* Gal) and acceptor (Gal *versus* GlcNAc) substrates, maintaining the same linkage specificity (β1,3-linkage).

During the course of study to isolate β3Gal-T1 homologs, we have identified three additional types of putative members of the β3Gal-T family. In this study, we show additional examples that glycosyltransferases structurally related to the β3Gal-T family exhibit β1,3-*N*-acetylglucosaminyltransferase activity, but not β1,3-galactosyltransferase activity. These results indicate that β1,3-*N*-acetylglucosaminyltransferases (β3Gn-Ts) form a family having structural similarity to the β3Gal-T family. Alignment of primary sequences of all members of the β3Gn-T and β3Gal-T families revealed that the members are clustered into four subgroups, probably reflecting enzymatic activity and substrate specificity. Transfection experiments and *in vitro* enzymatic analysis have demonstrated that β3Gn-T2, -T3, and -T4 are able to catalyze the initiation and elongation of poly-*N*-acetyllactosamine sugar chains; however, they exhibit different substrate specificity. These results, taken together with the different distributions of these enzymes, indicate that β3Gn-T2, -T3, and -T4 each exert distinct roles in physiological and pathological processes.

EXPERIMENTAL PROCEDURES

Nomenclature of β1,3-Galactosyltransferases and β1,3-N-Acetylglucosaminyltransferases—To simplify discussion, five members of the cloned human β3Gal-Ts will be called β3Gal-T1, -T2, -T3, -T4, and -T5 according to the designation of Kolbinger *et al.* (2), Clausen and co-workers (3), and Narimatsu and co-workers (4). Five types of β3Gn-Ts cloned to date will be referred to tentatively as follows. A β3Gn-T and newly isolated β3Gn-Ts in this study, which show structural similarity to the β3Gal-T family, will be called β3Gn-T1, -T2, -T3, -T4, and -T5. Another type of β3Gn-T, which was isolated by expression cloning using anti-i-antigen antibody (8) and showed no structural similarity to the β3Gal-T family, will be called iGnT according to Fukuda *et al.* (8).

Cell Lines—Namalwa KJM-1, a subline of the human Burkitt lymphoma cell line Namalwa, was cultivated in serum-free RPMI 1640 medium as described (9, 10). Cell lines SK-N-MC and Colo205 were obtained from the American Type Culture Collection. These cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum. Sf9 and Sf21 insect cells were cultured at 27 °C in TNM-FH insect medium (Pharmingen) as described previously (11).

Preparation of cDNA Libraries and Single Strand cDNAs—cDNA libraries of human gastric mucosa and human placenta were constructed as described previously (12). Single strand cDNAs were synthesized from total RNA prepared from the neuroblastoma cell line SK-N-MC.

Isolation of Human βGn-T2, -T3, and -T4 cDNAs—EST fragments encoding amino acid sequences similar to the human β3Gal-T1 sequence were retrieved from the EST division of the GenBank™/EBI Data Bank using the FrameSearch algorithm (Compugen) (Table I). The human β3Gn-T2 cDNA fragment (600 bp) was isolated from a human gastric mucosa cDNA library by PCR using primers 5′-CGGGACAGATTTAAAGACTTTCTGC-3′ and 5′-GTAGAGGCCAGAGTAAACAACTTCT-3′. The human β3Gn-T3 cDNA fragment (200 bp) was isolated from a human placenta cDNA library by PCR using primers 5′-CGTGGGGCAACTGATCCAAAACG-3′ and 5′-ACCCAGGAAGACATCATCAATGGG-3′. The PCR conditions were 99 °C for 10 min; followed by 30 cycles of 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min; and 72 °C for 10 min of incubation. The β3Gn-T2 and -T3 cDNA fragments were cloned into pT7Blue (Novagen) and sequenced. Digoxigenin-labeled probes were prepared from the above-mentioned 600- and 200-bp fragments using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals) and used to isolate the full-length cDNAs for β3Gn-T2 and -T3 by plaque hybridization. The full-length β3Gn-T2 cDNA (1.9 kb) was prepared from phage DNA by *Sal*I and *Xba*I digestion and subcloned into pBluescript II SK(+) to yield pBS-β3Gn T2. A plasmid (pBS-β3Gn T3) containing the full-length β3Gn T3 cDNA in pBluescript SK(−) was recovered by *in vivo* excision. The full-length β3Gn T4 cDNA (1.3 kb) was amplified from single strand cDNAs derived from the neuroblastoma cell line SK-N-MC by PCR using primers 5′-CACAGCCTGAGACTCATCTCGCT-3′ and 5′-AGGCATCAAATTTCGCATCACGATAG-3′ and was inserted into the pT7Blue-T vector to make pT7B-β3Gn T4.

TABLE I
List of the ESTs used to isolate β3Gn-T2, -T3, and -T4 cDNAs
GenBank™/EBI accession numbers of EST clones are indicated according to the direction of the nucleotide sequences.

β3Gn-T2		β3Gn-T3		β3Gn-T4	
5′	3′	5′	3′	5′	3′
H78875	N53174	R77875	R77780	AA133340	AA133381
H47990	N66915	R75815	R75816	R18612	R41690
W26453	N51037	W25364			
R31722	H93550				
H13125	H47991				
R74552	H80116				
R82783	G23485				

DNA Sequencing—DNA sequences were determined by the dideoxynucleotide chain termination method using an ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Inc.).

Construction of Plasmids for Expressing β3Gn T2, -T3, and -T4 in Animal Cells—A β3Gn T2 cDNA fragment (prepared from pBS-β3Gn T2 by *Sal*I and *Xba*I digestion, followed by blunting and addition of the *Sfi*I linker (5′-CTTTAGAGCA-3′ and 5′-CTCTAAAG-3′)) was inserted between the *Sfi*I sites of pAMo to yield pAMo-β3Gn T2. A β3Gn T3 cDNA fragment prepared from pBS-β3Gn T3 by *Hin*dIII and *Not*I digestion was inserted between the *Hin*dIII and *Not*I sites of pAMo to yield pAMo-β3Gn T3. A β3Gn T4 cDNA fragment (prepared from pT7B-β3Gn T4 by *Sma*I and *Hin*cII digestion, followed by addition of the *Sfi*I linker) was inserted between the *Sfi*I sites of pAMo to yield pAMo-β3Gn T4.

Expression of β3Gn T2, -T3, and -T4 in Namalwa KJM-1 Cells—Namalwa KJM-1 cells were transfected with pAMo-β3Gn T2, pAMo-β3Gn T3, or pAMo-β3Gn T4 by electroporation as described (9, 10) and grown for 24 h. Stably transfected cells were selected by cultivation for >14 days in the presence of G418 (0.5 mg/ml).

Flow Cytometric Analysis—Transfected Namalwa KJM-1 cells (5×10^6 cells) were incubated in 100 μl of phosphate-buffered saline for 60 min at 37 °C in the presence or absence of 20 milliunits of *Clostridium perfringens* neuraminidase (N2133, Sigma). These cells were stained with human anti-i-antigen serum (Den) (13), followed by fluorescein isothiocyanate-conjugated goat anti-human IgM, and were analyzed on a FACSCalibur apparatus (Becton Dickinson) as described (8). For lectin staining, cells were stained with 10 μg/ml fluorescein isothiocyanate-labeled *Lycopersicon esculentum* pokeweed mitogen (LEA) or *agglutinin* (PWM; both from EY Laboratories).

Construction and Purification of β3Gn-T Proteins Fused to the FLAG Peptide—The putative catalytic domain of each β3Gn-T2, -T3, and -T4 was expressed as a secreted protein fused to the FLAG peptide in insect cells. A 1.1-kb DNA fragment encoding a COOH-terminal portion of β3Gn-T2 (amino acids 21–297) was amplified by PCR using primers 5′-CACGGATCCAGCCAAGAAAAAAATGGAAAAGGGGA-3′ and 5′-ATCCGATAGCGGCCGCTTAGCATTTTAAATGAGCACTCTGCAAC-3′, digested with *Bam*HI and *Not*I, and inserted between the *Bam*HI and *Not*I sites of pVL1393-F2 to yield pVL1393-F2G2. pVL1393-F2 is an expression vector derived from pVL1393 (Pharmingen) and contains a fragment encoding the signal peptide of human immunoglobulin κ (MHFQVQIFSFLLISASVIMSRG) and the FLAG peptide (DYKDDDDK). Joining in-frame a cDNA fragment with a unique *Bam*HI site of pVL1393-F2 just downstream of the COOH terminus of the FLAG peptide enables the cDNA product to be secreted as a protein fused to the FLAG peptide. A 1.0-kb DNA fragment encoding a COOH-terminal portion of β3Gn-T3 (amino acids 38–372) was amplified by PCR using primers 5′-CGCGGATCCTCCCCACGGTCCGTGGACCAG-3′ and 5′-ATAGTTTAGCGGCCGCGGAAGGGCTCAGCAGCGTCG-3′, digested with *Bam*HI and *Not*I, and inserted between the *Bam*HI and *Not*I sites of pVL1393-F2 to yield pVL1393-F2G3. A 0.9-kb DNA fragment encoding a COOH-terminal portion of β3Gn-T4 (amino acids 56–378) was amplified by PCR using primers 5′-ATAAGATCTGCAGGAGACCCCACGGCCCACC-3′ and 5′-ATAGTTATGCGGCCGCCTCAGGCTGTTGC-

CCAACCCAC-3′, digested with *Bgl*II and *Not*I, inserted between the *Bam*HI and *Not*I sites of pVL1393-F2 to yield pVL1393-F2G4. The PCR-amplified portions of pVL1393-F2G2, pVL1393-F2G3, and pVL1393-F2G4 were sequenced to confirm the absence of possible PCR errors.

Sf9 insect cells were cotransfected with BaculoGold viral DNA (Pharmingen) according to the manufacturer's instruction and each of plasmids pVL1393-F2G2, pVL1393-F2G3, and pVL1393-F2G4 and were incubated for 3 days at 27 °C to produce individual recombinant viruses. These viruses were amplified three times to reach titers of $\sim 10^9$ plaque-forming units/ml. Sf21 insect cells (4×10^7 cells; Pharmingen) were infected at a multiplicity of 10 and incubated in 30 ml of TNM-FH insect medium at 27 °C for 72 h to yield conditioned medium including recombinant β3Gn-T proteins fused to the FLAG peptide, which were readily purified by anti-FLAG M1 antibody resin (Sigma) according to the protocol of the manufacturer. Briefly, the culture medium (30 ml) was collected by centrifugation and added to NaCl (150 mM final concentration), NaN_3 (0.1% final concentration), and M1 antibody resin (30 μl) to adsorb the recombinant β3Gn-T proteins on the resin. The resin was recovered by centrifugation and washed three times with buffer (1 ml) consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$. The recombinant β3Gn-T proteins were eluted with buffer (90 μl) consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA, followed by the addition of $CaCl_2$ (4 mM final concentration), and stored at 4 °C until use. The amount of the purified proteins was not enough for accurate quantification.

Silver Staining and Western Blot Analysis—The enzymes purified above (3 μl) were subjected to SDS-polyacrylamide gel electrophoresis, followed by silver staining or Western blot analysis. Silver staining was performed using a silver staining kit (Wako Bioproducts). Proteins separated on 6% SDS-polyacrylamide gel were transferred to an polyvinylidene difluoride membrane (Immobilon, Millipore Corp.) in a Trans-Blot SD cell (Bio-Rad). The membrane was blocked with phosphate-buffered saline containing 5% skim milk at 4 °C overnight and then incubated with 10 μg/ml M2 antibody (Sigma). The membrane was stained with ECL Western blot detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Glycosyltransferase Assays and Product Characterization—The *N*-acetylglucosaminyltransferase activities of the purified proteins (15 μl) were assayed in 50 mM MOPS (pH 7.5), 5 mM $MgCl_2$, 5 mM UDP-GlcNAc, and 10 mM unlabeled acceptors (a total volume of 40 μl). The following oligosaccharides were used as acceptors: lactose (Galβ1–4Glc), *N*-acetyllactosamine (Galβ1–4GlcNAc), lacto-*N*-neotetraose (LNnT; Galβ1–4GlcNAcβ1–3Galβ1–4Glc), *p*-lacto-*N*-neohexaose (*p*-LNnH; Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc), and lacto-*N*-tetraose (LNT; Galβ1–3GlcNAcβ1–3Galβ1–4Glc). LNnT and LNT were purchased from Oxford Glycosystems. Lactose and *p*-LNnH were purchased from Sigma. Parallel reactions were done in the absence of UDP-GlcNAc to identify products. After incubation at 37 °C for the appropriate times (2 h for β3Gn-T2 and 16 h for β3Gn-T3 and -T4), the reactions were terminated by boiling. After centrifugation, the reaction mixtures were analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE/PAD, Dionex Corp.). CarboPac PA10 was used as a column, and elution was performed with a gradient of 40–125 mM NaOH in 30 min at a flow rate of 1 ml/min. The structures of the reaction products derived from lactose and LNnT were confirmed by comparison of their retention times on HPLC with those of the standard oligosaccharides GlcNAcβ1–3Galβ1–4Glc and GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc, which were prepared from LNnT and *p*-LNnH by digestion with jack bean β-galactosidase.

To further confirm the structure of the reaction product derived from lactose, it was digested with endo-β-galactosidase or modified by β1,4-galactosyltransferase. To avoid the inhibitory effect of MOPS on endo-β-galactosidase, an *N*-acetylglucosaminyltransferase assay was done without MOPS using lactose as an acceptor. The reaction was stopped by boiling, and the reaction mixture was recovered by centrifugation. The reaction mixture (15 μl) was added to acetate buffer (50 mM final concentration; pH 5.8) and incubated with 250 milliunits/ml *Escherichia freundii* endo-β-galactosidase (Seikagaku Kogyo) (14) in a total volume of 41 μl at 37 °C for 16 h, followed by analysis with HPAE/PAD as described above. Alternatively, the reaction mixture (15 μl) was added to Tris-HCl (50 mM final concentration; pH 8.0) and incubated with 750 milliunits/ml bovine milk β1,4-galactosyltransferase (Sigma) in a total volume of 40 μl at 37 °C for 16 h, followed by analysis with HPAE/PAD as described above.

Endo-β-galactosidase digestion of the product yielded two peaks that comigrated with the standard oligosaccharides GlcNAcβ1–3Galβ and glucose at a 1:1 molar ratio. These results clearly indicated that the product derived from lactose was GlcNAcβ1–3Galβ1–4Glc.

Since the amount of the purified proteins was not enough for accurate quantification, enzymatic activity is defined as picomoles of acceptor substrate *N*-acetylglucosaminylated per ml of culture medium/h. The amounts of reaction products were determined from their absorbance intensities using individual standards.

Alternatively, the *N*-acetylglucosaminyltransferase activities of the purified proteins (15 μl) were assayed in 200 mM MOPS (pH 7.5), 20 mM $MgCl_2$, 20 mM UDP-GlcNAc, and 50 μM pyridylaminated acceptors (total volume of 80 μl). As acceptors, the following pyridylaminated oligosaccharides were used: LNnT, lacto-*N*-fucosylpentaose (LNFP) III (Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc), LNT, LNFP-II (Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4Glc), LNFP-V (Galβ1–3GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc), and lacto-*N*-difucosylhexaose II (Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc). After incubation at 37 °C for the appropriate times (2 h for β3Gn-T2 and 15 h for β3Gn-T3 and -T4), the reactions were terminated by boiling and analyzed by HPLC as described previously, with exception that HPLC was performed at 50 °C with a flow rate of 0.5 ml/min (9, 10). Parallel reactions were done in the absence of UDP-GlcNAc to identify products and to check hydrolysis of substrate and product. The oligosaccharides were purchased from Oxford Glycosystems and pyridylaminated according to the method of Kondo *et al.* (15). The amounts of products were determined from their fluorescence intensities using pyridylaminated lactose as a standard.

The reaction product derived from pyridylaminated LNnT was identified by comparison of the retention time on HPLC with that of the pyridylaminated standard oligosaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc, which was prepared from pyridylaminated *p*-LNnH by digestion with jack bean β-galactosidase. To further confirm the structure of the reaction product, the reaction product was modified by β1,4-galactosyltransferase to examine whether *p*-LNnH was produced or not. The reaction mixtures (20 μl) was incubated with 20 milliunits of bovine milk β1,4-galactosyltransferase in the presence or absence of UDP-Gal (20 mM) in a total volume of 30 μl at 37 °C for 15 h according to manufacturer's recommendations. The product further modified by β1,4-galactosyltransferase comigrated with pyridylaminated *p*-LNnH on HPLC. These results indicated that the product was pyridylaminated GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc. The galactosyltransferase activities of the purified proteins (15 μl) were assayed using pyridylaminated oligosaccharides (GlcNAcβ1–3Galβ1–4Glc and LNnT) as substrates as described previously (4).

Preparation and Fractionation of Blood Leukocytes—Human polymorphonuclear leukocytes, monocyte-enriched population, and lymphocyte-enriched population were obtained as described previously (10).

Quantitative Analysis of the Three β3Gn-T Transcripts in Human Tumor Cell Lines and Human Tissues by Competitive RT-PCR—The levels of the β3Gn-T2, -T3, and -T4 transcripts were measured by competitive RT-PCR as described in detail previously (4, 16). Competitor DNA plasmids carrying a small deletion within the respective cDNA were constructed by appropriate restriction endonuclease digestion as shown in Table II. For instance, a competitor DNA plasmid for measuring β3Gn-T2 transcripts was prepared by deleting the 227-bp *Eco*81I-*Pfl*MI fragment in β3Gn-T2 cDNA from the standard DNA plasmid pBS-β3Gn T2.

Single strand cDNAs were synthesized with an oligo(dT) primer from 6 μg of DNase I-treated total RNA from human tissues (colon, jejunum, stomach body, stomach antrum, and esophagus) and cell lines (HL-60 and Colo205) as described previously (4). Single strand cDNAs from human leukocytes were prepared as described (10). In addition, single strand cDNAs were synthesized with an oligo(dT) primer from 1 μg of poly(A)$^+$ RNAs from 35 human tissues (CLONTECH) using a Superscript™ pre-amplification system for first strand cDNA synthesis (Life Technologies, Inc.) according to manufacturer's instructions. After cDNA synthesis, the reaction mixture was diluted 50-fold with H_2O and then stored at −80 °C until use.

Competitive RT-PCR was performed with AmpliTaq Gold™ (PerkinElmer Life Sciences). The annealing temperatures and specific primers used are listed in Table II. The amount of each of the β3Gn-T transcripts was normalized by the amount of β-actin transcripts (4, 16).

Determination of Chromosomal Localization—The chromosomal localizations of the β3Gn-T2, -T3, and -T4 genes were determined using 3′-EST mapping data (NCBI Protein Database). The chromosomal localization of the β3Gal-T1 gene was determined by PCR analysis using a series of genomic DNAs from hamster-human somatic hybrids (BIOSMAP™ Somatic Cell Hybrid PCRable™ DNAs, BIOS Laboratories)

TABLE II
Oligonucleotide primers and conditions used for competitive RT-PCR analysis

Target gene	Primer sets	Sizes of PCR products		Restriction enzymes for competitor DNA	Annealing temperature
		Target	Competitor		
		bp			°C
β3Gn-T2					
Forward	5′-GAGAAGTTCTGGAAGATATCTACC-3′	646	419	*Eco*81I-*Pfl*MI	55
Reverse	5′-CTATTCAAGTAATTCAGGATGTGA-3′				
β3Gn-T2					
Forward	5′-GTGCCATGCCAACACCTCTATGGT-3′	498	312	*Bst*EII-*Pml*I	65
Reverse	5′-TCCTGCAGGTAGAAGACCATGTTG-3′				
β3Gn-T4					
Forward	5′-GTCTCTTCTTGACCTATCGTCACT-3′	619	399	*Tth*111I-*Nar*I	65
Reverse	5′-AGTTCAGCATCTTCCATGATAGCC-3′				

and specific primers (5′-TTCAGCCACCTAACAGTTGCCAGG-3′ and 5′-ATACCTTCTTCGTGGCTTGGTGGAG-3′). The predicted fragment of 495 bp was amplified only when genomic DNA from a hybrid containing human chromosome 2 (hybrid 852) was used, indicating that this gene is located on chromosome 2.

RESULTS

Identification and Isolation of β3Gn-T2, -T3, and -T4—A homology search in the EST division of the GenBank™/EBI Data Bank using the FrameSearch algorithm revealed the existence of six types of cDNAs encoding proteins with low but significant similarity to β3Gal-T1, three of which have been reported recently to be β3Gal-T2, -T3, and -T4 (2, 3). Based on the nucleotide sequence of the ESTs shown in Table I, we prepared specific probes and isolated three types of full-length cDNAs encoding novel proteins (designated β3Gn-T2, -T3, and -T4) of 397, 372, and 378 amino acid residues, respectively, with structural similarity to β3Gal-T1. β3Gn-T2 and -T3 cDNAs were obtained from human gastric mucosa, and β3Gn-T4 cDNA was from the neuroblastoma cell line SK-N-MC.

A Kyte-Doolittle hydropathy analysis (17) revealed that β3Gn-T2, -T3, and -T4 show type 2 transmembrane topology typical of most glycosyltransferases. β3Gn-T2 is predicted to consist of an N-terminal cytoplasmic domain of 9 residues, a transmembrane segment of 19 residues, and a stem region and catalytic domain of 369 residues. β3Gn-T3 is predicted to consist of an N-terminal cytoplasmic domain of 11 residues, a transmembrane segment of 21 residues, and a stem region and catalytic domain of 340 residues. The predicted coding region of β3Gn-T4 has two potential initiation codons, both of which are in agreement with Kozak's rule (18). Therefore, it is predicted that β3Gn-T4 is composed of two different N-terminal cytoplasmic domains of 29 and 4 residues, a transmembrane segment of 20 residues, and 329 residues containing the stem region and catalytic domain (Fig. 1*a*).

Fig. 1*A* shows a multiple alignment of the amino acid sequences of β3Gn-T2, -T3, and -T4 as well as β3Gn-T1 and five members of the β3Gal-T family. β3Gn-T2, -T3, and -T4 show 19–24, 22–26, and 22–25% identities, respectively, to the β3Gal-T family (β3Gal-T1, -T2, -T3, -T5, and -T5), whereas they show 15, 18, and 15% identities to β3Gn-T1. β3Gn-T2, -T3, and -T4 show 40–45% identity one another. The sequence similarities are limited to the putative catalytic regions. Several sequence motifs conserved in the β3Gal-T family are also shared by β3Gn-T2, -T3, and -T4 as well as β3Gn-T1. Twenty-five amino acid residues located separately in the putative catalytic regions are identical among all the proteins. Three cysteine residues conserved in all members of the β3Gal-T family are also maintained in β3Gn-T2, -T3, and -T4, whereas two of these are not conserved in β3Gn-T1 (Fig. 1*A*, *white arrows*), indicating that β3Gn-T1 is relatively distinct from other members, especially in the context of the three-dimensional structure. There are five potential *N*-linked glycosylation sites in β3Gn-T2, three in β3Gn-T3, and three in β3Gal-T4. One site in a highly conserved motif is maintained among all the proteins (Fig. 1*A*, *black arrow*). The phylogenetic tree of these proteins generated using the amino acid sequences of the putative catalytic domains demonstrates that β3Gn-T2, -T3, and -T4 form a subgroup, indicating that they have similar enzymatic activity (Fig. 1*B*).

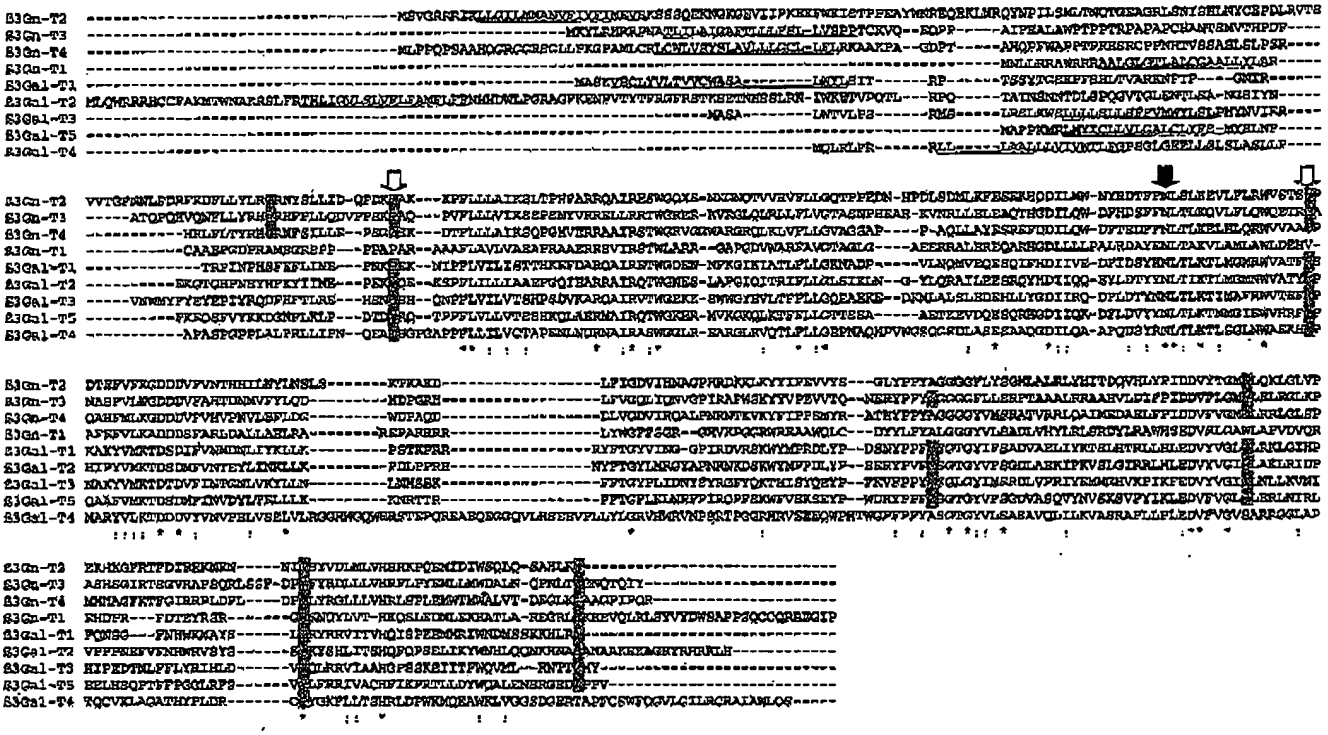
Production of Secreted Recombinant Proteins Fused to the FLAG Peptide—To examine the enzymatic activities of β3Gn-T2, -T3, and -T4, we expressed the putative catalytic domain of each enzyme (amino acids 31–397 of β3Gn-T2, amino acids 38–372 of β3Gn-T3, and amino acids 56–378 of β3Gn-T4) as a secreted protein fused to the FLAG peptide in Sf21 insect cells. The FLAG-fused recombinant proteins were partially purified using anti-FLAG M1 antibody resin and analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining (Fig. 2*A*) or Western blotting using anti-FLAG monoclonal antibody (Fig. 2*B*). Two major bands with apparent molecular masses of 45.5 and 48 kDa, broad bands of 42–45 kDa, and two major bands of 37.6 and 40 kDa were observed specifically for β3Gn-T2, -T3, and -T4, respectively. The FLAG-fused recombinant proteins for β3Gn-T2, -T3, and -T4 have predicted molecular masses of 43,674, 39,507, and 37,608 Da for the respective polypeptides, indicating glycosylation of the recombinant proteins produced by insect cells.

β3Gn-T2, -T3, and -T4 Are β1,3-N-Acetylglucosaminyltransferases—The glycosyltransferase activities of the partially purified FLAG-fused recombinant proteins were examined. When lactose was used as an acceptor, β3Gn-T2, -T3, and -T4 showed a significant amount of *N*-acetylglucosaminyltransferase activity, whereas no activity was detected in a sample prepared from the conditioned medium of insect cells infected with empty vector virus. The structure of the product was estimated to be GlcNAcβ1–3Galβ1–4Glc by comparing the retention time on HPLC with that of the standard oligosaccharide (Fig. 3*A*). To further confirm the structure of the product, it was digested with endo-β-galactosidase or modified by β1,4-galactosyltransferase. Digestion of the product by *E. freundii* endo-β-galactosidase yielded two peaks comigrating with the standard oligosaccharides GlcNAcβ1–3Gal and glucose at a 1:1 molar ratio (Fig. 3, compare *A* and *B*). Modification of the product by bovine milk β1,4-galactosyltransferase yielded a peak comigrating with LNnT (Fig. 3, compare *A* and *C*). These results clearly indicated that the product was GlcNAcβ1–3Galβ1–4Glc.

On the other hand, β3Gn-T2, -T3, and -T4 showed no β1,3-galactosyltransferase activity for GlcNAcβ1–3Galβ1–4Glc or LNnT. Taken together, β3Gn-T2, -T3, and -T4 were demonstrated to be novel β1,3-*N*-acetylglucosaminyltransferases.

Substrate Specificity of β3Gn-T2, -T3, and -T4—Analysis of the substrate specificity of β3Gn-T2, -T3, and -T4 revealed that these enzymes utilized common oligosaccharides as substrates, but the substrate preference was significantly different (Tables III and IV). β3Gn-T2 and -T4 showed more preferential activity for LNnT than for LNT, which is consistent with the nature of

A



B

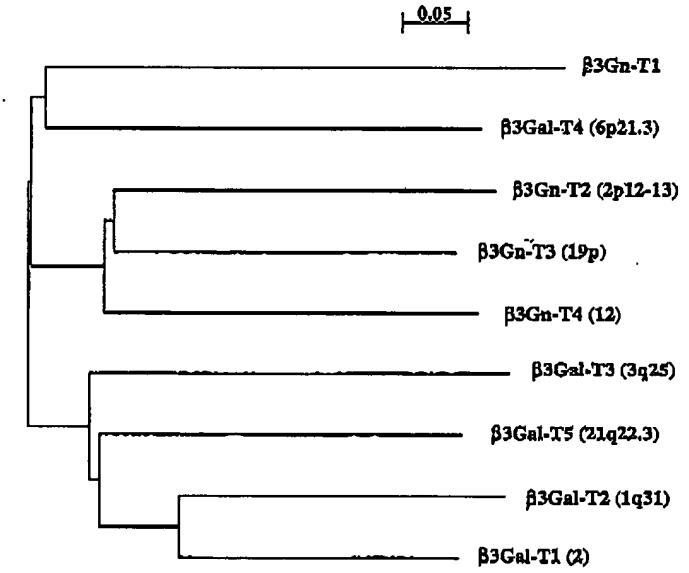


FIG. 1. **Comparison of novel β3Gn-Ts with β3Gn-T1 and the β3Gal-T family.** *A*, multiple sequence analysis (ClustalX) of the β3Gn-T family (β3Gn-T1, -T2, -T3, and -T4) and the β3Gal-T family (β3Gal-T1, -T2, -T3, -T4, and -T5). Introduced gaps are shown by *hyphens*. The putative transmembrane domains are *underlined*. *Asterisks* indicate identical amino acids in all proteins. Conserved amino acids are shown by *colons*. Cysteine residues conserved in all the proteins or subgroups are *shaded*. The *white arrows* indicate cysteine residues conserved in all the proteins except for β3Gn-T1. The *black arrow* indicates the conserved possible *N*-glycosylation site. *B*, phylogenetic tree of the β3Gn-T and β3Gal-T families. The phylogenetic tree was produced with ClustalX presented in *A* using amino acid sequences of the predicted catalytic domains. The chromosomal localizations of the respective genes, except for the β3Gn-T1 gene, are indicated in *parentheses* (Refs. 4 and 49 and this study).

β3Gn-T1 and iGnT (7, 8). In contrast, β3Gn-T3 utilized LNT as a substrate comparable to LNnT. In common, fucosylation at the penultimate GlcNAc residue in LNnT or LNT yielded poor substrates (LNFP-III, LNFP-II, and lacto-*N*-difucosylhexaose II). LNFP-V, which is an LNT derivative fucosylated at the reducing terminal glucose residue, was a relatively good substrate for β3Gn-T2 compared with LNT.

β3Gn-T2 transferred GlcNAc efficiently to both lactose and *p*-LNnH, as well as LNnT, whereas the relative activity *N*-acetyllactosamine was 21% compared with that of LNnT. β3Gn-T3 preferred lactose (235% relative activity) as a substrate, followed by LNnT (100%) and *p*-LNnH (45%), whereas it showed no activity for *N*-acetyllactosamine. β3Gn-T4 showed 33, 9, and 0% relative activities for lactose, *N*-acetyllac-

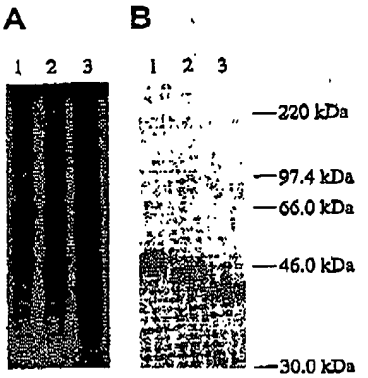


FIG. 2. **SDS-polyacrylamide gel electrophoresis analysis of the secreted recombinant enzymes.** The FLAG-fused secreted proteins for β3Gal-T2 (*lanes 1*), -T3 (*lanes 2*), and -T3 (*lanes 3*) were purified by anti-FLAG antibody resin and subjected to SDS-polyacrylamide gel electrophoresis, followed by silver staining (*A*) or Western blotting using anti-FLAG antibody M2 (*B*).

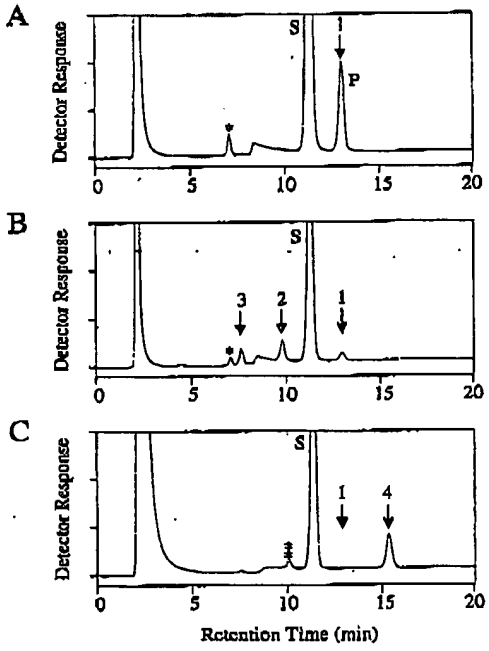


FIG. 3. **HPLC analysis of the reaction product generated from lactose by recombinant β3Gn-T2.** *A*, the *N*-acetylglucosaminyltransferase activity of the purified β3Gn-T2 protein was assayed using lactose (Galβ1–4Glc) as an acceptor. The reaction mixture was analyzed using high-pH anion-exchange chromatography with pulsed amperometric detection. The peaks for substrate lactose and the generated product are labeled *S* and *P*, respectively. *Arrow 1* indicates the elution position of the standard oligosaccharide GlcNAcβ1–3Galβ1–4Glc. Based on the elution position, the peak indicated by the *asterisk* seems to be GlcNAc, which may be a degradation product of UDP-GlcNAc. The peak with a retention time of 2–3 min may be glycerol, which appeared in the absence of UDP-GlcNAc. *B*, the reaction mixture described in *A* was analyzed after digestion with endo-β-galactosidase. *Arrows 2* and *3* indicate the elution positions of the standard oligosaccharides GlcNAcβ1–3Gal and glucose, respectively. *C*, the reaction mixture described in *A* was analyzed after galactosylation with β1,4-galactosyltransferase. *Arrow 4* indicates the elution position of the standard oligosaccharide LNnT (Galβ1–4GlcNAcβ1–3Galβ1–4Glc). Based on the elution position, the peak indicated by the *double asterisks* seems to be Galβ1–4GlcNAc, which would be a galactosylation product of GlcNAc indicated by the *asterisk* in *A*.

tosamine, and *p*-LNnH, respectively, compared with LNnT (100%). β3Gn-T1 was reported to efficiently utilize *N*-acetyllactosamine as well as lactose (Table III), showing a significant difference in substrate specificity compared with β3Gn-T2, -T3, and -T4.

β3Gn-T2, -T3, and -T4 Each Direct Biosynthesis of Poly-N-acetyllactosamines in Namalwa KJM-1 Cells—To examine the *in vivo* enzymatic activities of β3Gn-T2, -T3, and -T4, Namalwa KJM-1 cells were transfected with pAMo-β3Gn T2, pAMo-β3Gn T3, pAMo-β3Gn T4, or the empty vector pAMo and examined for changes in the surface expression of the poly-*N*-acetyllactosamine sugar chains by flow cytometric analyses using the anti-i-antigen antibody (Den) and specific lectins (LEA and PWM).

Since Den, LEA, and PWM are likely to recognize non-sialylated poly-*N*-acetyllactosamines more preferentially than sialylated ones, the transfected cells were treated with neuraminidase before staining. As shown in Fig. 4, expression of β3Gn-T2, -T3, or -T4 increased the levels of poly-*N*-acetyllactosamines recognized by Den, LEA, or PWM compared with the vector (pAMo) transfectant, consistent with *in vitro* enzymatic activity. In particular, expression of β3Gn-T3 or -T4 led to a remarkable increase in reactivity to Den, in contrast to the slight increase in the β3Gn-T2 transfectant. On the other hand, reactivity to LEA or PWM was increased in the β3Gn-T2 transfectant more clearly than in the other two transfectants. These results indicate that β3Gn-T2, -T3, and -T4 each are involved in the biosynthesis of poly-*N*-acetyllactosamine sugar chains in transfected cells.

Expression Levels of the β3Gn-T2, -T3, and -T4 Transcripts—The expression levels of the β3Gn-T2, -T3, and -T4 transcripts were examined by competitive RT-PCR. These genes were differentially expressed in human tissues and cells (Table V). β3Gn-T2 was ubiquitously expressed in the tissues and cells tested, but expression of β3Gn-T3 and -T4 was relatively restricted. β3Gn-T3 was expressed in colon, jejunum, stomach (body and antrum), esophagus, placenta, and trachea. β3Gn-T4 was mainly expressed in brain tissues such as whole brain, hippocampus, amygdala, cerebellum, and caudate nucleus, as well as in colon, esophagus, and kidney.

β1,3-*N*-Acetylglucosaminyltransferase activities were detected in several tissues, cells, and sera, some of which were characterized using partially purified enzymes (19–28). Poly-*N*-acetyllactosamines are known to serve as backbone oligosaccharides for presenting the sialyl-Le^x and sialyl-Le^a determinants, which function as selectin ligands in leukocytes and several cancer cells such as colon cancer cells (29–40). The human promyelocytic leukemia cell line HL-60 and the human colon adenocarcinoma cell line Colo205 are known to express β1,3-*N*-acetylglucosaminyltransferase activities as well as poly-*N*-acetyllactosamines presenting the sialyl-Le^a and sialyl-Le^x determinants. Therefore, it was of significant interest to examine the expression levels of β3Gn-T2, -T3, and -T4 in leukocytes and cancer cells such as HL-60 and Colo205. β3Gn-T2, but not β3Gn-T3 and -T4, was significantly expressed in HL-60 and Namalwa KJM-1 cells (Table V) as well as in human peripheral polymorphonuclear cells and lymphocytes (data not shown). On the other hand, β3Gn-T2 and -T3, but not β3Gn-T4, were highly expressed in the colon cancer cell line Colo205 (Table V).

DISCUSSION

In this study, we identified three novel β1,3-*N*-acetylglucosaminyltransferases (β3Gn-T2, -T3, and -T4) that show structural similarity to β3Gn-T1 as well as the β3Gal-T family, including five members (β3Gal-T1, -T2, -T3, -T4, and T5), demonstrating the existence of a β3Gn-T family now consisting of four members (β3Gn-T1, -T2, -T3, and -T4). The existence of the multiple enzymes showing similar activity is a common feature of glycosyltransferases, which was demonstrated for

TABLE III

Analysis of substrate specificity of β3Gn-T2, -T3, and -T4 using pyridylaminated oligosaccharides as substrates

Assay conditions were described under "Experimental Procedures." Activities are expressed as a percentage of the activity for pyridylaminated LNnT. The activities of β3Gn-T2, -T3, and -T4 for pyridylaminated LNnT were 330, 7.1, and 1.9 pmol/ml of medium/h, respectively. LNDFH-II, lacto-*N*-difucosylhexaose II.

Substrate	Structure	Relative activity		
		β3Gn-T2	β3Gn-T3	β3Gn-T4
			%	
LNnT	Galβ1–4GlcNAcβ1–3Galβ1–4Glc	100	100	100
LNFP-III	Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc	0	5	7
LNT	Galβ1–3GlcNAcβ1–3Galβ1–4Glc	4	85	8
LNFP-II	Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4Glc	0	0	0
LNFP-V	Galβ1–3GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc	31	65	8
LNDFH-II	Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc	0	0	0

TABLE IV

Analysis of substrate specificity of β3Gn-T2, -T3, and -T4 using unlabeled oligosaccharides as substrates

Assay conditions were described under "Experimental Procedures." Activities are expressed as a percentage of the activity for LNnT. The activities of β3Gn-T2, -T3, and -T4 for LNnT were 4900, 230, and 420 pmol/ml of medium/h, respectively. The activity of β3Gn-T1 was quoted from Ref. 7. LacNAc, *N*-acetyllactosamine; ND, not determined.

Substrate	Structure	Relative activity			
		β3Gn-T2	β3Gn-T3	β3Gn-T4	β3Gn-T1
			%		
LNnT	Galβ1–4GlcNAcβ1–3Galβ1–4Glc	100	100	100	100
Lactose	Galβ1–4Glc	128	235	33	67
LacNAc	Galβ1–4GlcNAc	21	0	9	96
LNT	Galβ1–3GlcNAcβ1–3Galβ1–4Glc	27	114	0	6
p-LNnH	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc	132	45	0	ND

Gal α2,3-sialyltransferases I–VI (9, 41–47, 49–52), GalNAc α2,6-sialyltransferases I–VI (53–58), NeuAc α2,8-sialyltransferases I–V (59–69), α1,3-fucosyltransferases III–VII and IX (10, 70–75, 77, 78)), core 2 β1,6-*N*-acetylglucosaminyltransferases 1–3 (79–82), large I β1,6-*N*-acetylglucosaminyltransferases (83), polypeptide *N*-acetylgalactosaminyltransferases (84–91), β1,4-galactosyltransferases 1–7 (92–100), and β1,3-galactosyltransferases 1–5 (1–4). Discovery of the β3Gn-T family in this study has clearly demonstrated a new feature of glycosyltransferases, that the β3Gn-T and β3Gal-T families show structural similarity despite of differences in both the transfer sugar (GlcNAc *versus* Gal) and the acceptor sugar (Gal *versus* GlcNAc).

We constructed the secreted recombinant proteins for β3Gn-T2, -T3, and -T4 fused to the FLAG peptide. Western blot analysis using anti-FLAG antibody revealed that the secreted enzymes were successfully produced by insect cells and were readily recovered by anti-FLAG M1 antibody resin. The FLAG-fused proteins adsorbed to the resin were eluted under mild conditions using buffer containing 2 mM EDTA. Since the eluted proteins showed activity comparative to that of the adsorbed proteins, it was confirmed that EDTA treatment did not damage the enzymes (data not shown). The molecular masses of the recovered proteins were equal to or larger than the predicted ones for their polypeptides, indicating some glycosylation and no significant degradation of the recovered proteins.

All of the recombinant proteins showed Gal β1,3-*N*-acetylgalactosaminyltransferase activity for common oligosaccharides, whereas their substrate preference was significantly different. Since the amount of the recombinant proteins used in this study was not enough for determination of the protein concentration, we could not precisely compare the relative activities of the enzymes. However, the relative activities of β3Gn-T2 for LNnT, lactose, Galβ1–4GlcNAc, and *p*-LNnH seemed to be higher than those of other enzymes (Tables III and IV). Considering the variety of acceptor substrates and the different reactivities of the transfected cells to anti-i-antigen antibody or PWM and LEA lectins, the higher activity of β3Gn-T2 for these oligosaccharides may reflect substrate specificity.

To date, β3Gn-T activities have been detected in several tissues, cells, and sera, some of which were characterized using partially purified enzymes (19–28). Based on the substrate specificity, β3Gn-T1, but not β3Gn-T2, -T3, and -T4, may correspond to a β3Gn-T partially purified from calf serum. β3Gn-T2 and -T4 showed more preferential activity for LNnT than for LNT, which was similar to the nature of the calf serum enzyme as well as β3Gn-T1 and iGnT (7, 8, 26); however, β3Gn-T2 and -T4 were distinguished from the calf serum enzyme and β3Gn-T1 by the activities for lactose (Galβ1–4Glc) and *N*-acetyllactosamine (Galβ1–4GlcNAc) (Table IV). On the other hand, β3Gn-T3 is quite unique since it showed activity for LNT comparable to LNnT. It has been reported that human colon cancer tissues and the colon cancer cell line Colo205 contain cancer-associated glycosphingolipids with dimeric Le^a antigens (Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–3(Fucα1–4)GlcNAc) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, β3Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le^a (Galβ1–3GlcNAcβ1–3Galβ1–3GlcNAc). On the other hand, it is difficult to ascribe the β3Gn-T activities detected in crude samples to the isolated β3Gn-Ts (β3Gn-T1, -T2, -T3, and -T4 or iGnT) because of the following reasons: differences in experimental conditions such as substrates used, the possibility of the existence of multiple β3Gn-Ts in the crude samples, and the possibility of the existence of additional unidentified β3Gn-Ts.

Analysis of substrate specificity revealed that β3Gn-T2, -T3, and -T4 each could be involved in the initiation and elongation of poly-*N*-acetyllactosamine synthesis by itself, which was demonstrated by increased expression of poly-*N*-acetyllactosamines in the transfected cells. The different reactivities of the respective transfectants to the anti-i-antigen antibody or LEA and PWM lectins may reflect the preference of the antibody and lectins as well as substrate specificity of these enzymes. On the other hand, expression of two or more β3Gn-Ts in the same cell, which was clearly demonstrated for Colo205 cells, indicates that poly-*N*-acetyllactosamine sugar chains might be synthe-

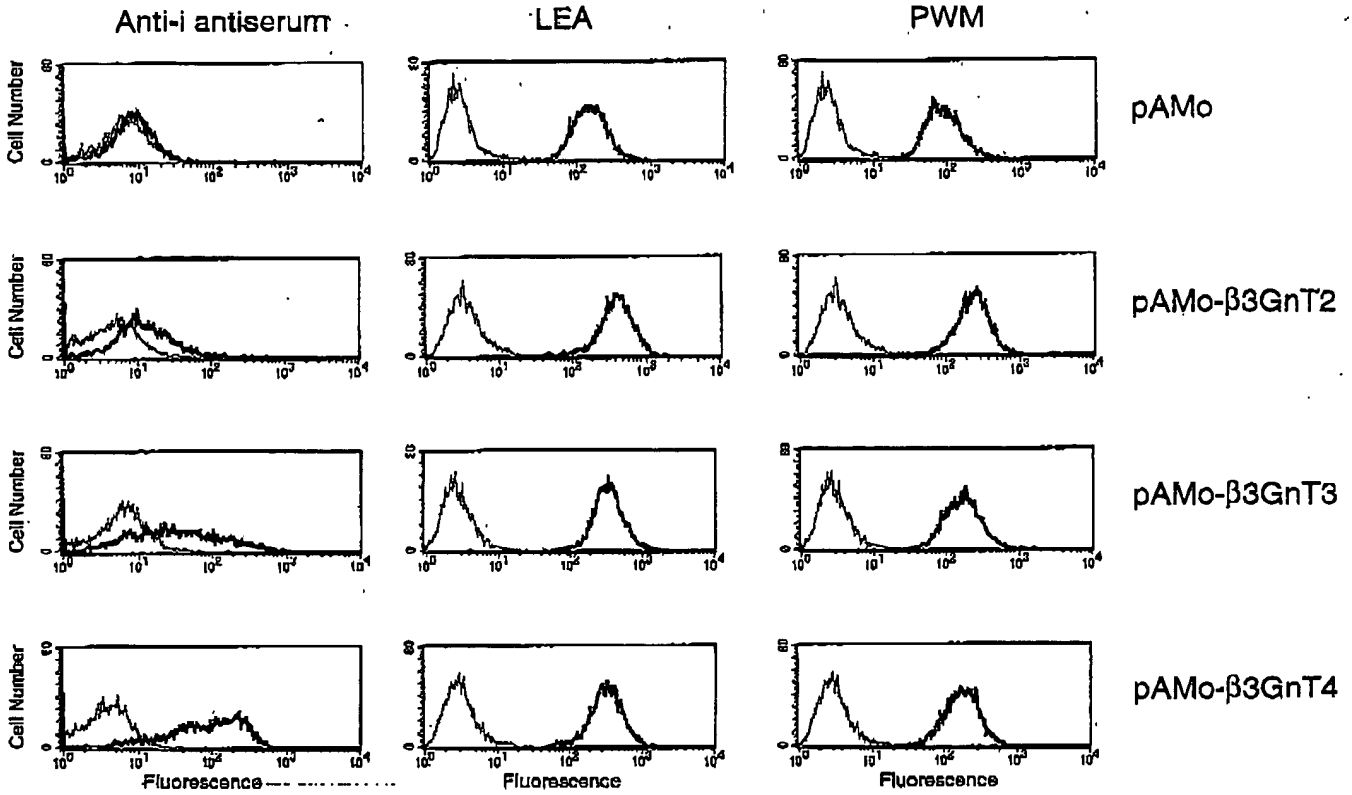


FIG. 4. **Flow cytometric analysis of Namalwa KJM-1 cells stably transfected with β3Gn-T2, -T3, or -T4 cDNA.** Namalwa KJM-1 cells were stably transfected with plasmid pAMo-β3Gn T2, pAMo-β3Gn T3, or pAMo-β3Gn T4, which directs expression of β3Gn-T2, -T3, or -T4, respectively, or with the empty vector pAMo. These cells were stained with anti-i-antigen antiserum or poly-*N*-acetyllactosamine-recognizing lectins (LEA or PWM) and subjected to flow cytometric analysis as described under "Experimental Procedures" (*thick lines*). As controls, the transfectants were stained with phosphate-buffered saline (*thin lines*).

sized by the concerted action of multiple β3Gn-Ts. Since Namalwa KJM-1 cells express endogenous β3Gn-T2, at least two β3Gn-Ts may be involved in the biosynthesis of poly-*N*-acetyllactosamines in the β3Gn-T3 or β3Gn-T4 transfectants.

The phylogenetic analysis using the amino acid sequences of the putative catalytic domains indicated that the members of the β3Gal-T and β3Gn-T families are clustered into the following four subgroups: β3Gal-T1, -T2, -T3, and -T5; β3Gn-T2, -T3, and -T4; β3Gal-T4; and β3Gn-T1 (Fig. 1*b*). Whereas β3Gal-T1, -T2, -T3, and -T5 catalyze the formation of the Galβ1–3GlcNAc structure, β3Gal-T4 forms the Galβ1–3GalNAc structure, indicating that enzymatic activity may reflect structural similarity. β3Gn-T1 was reported to have β3Gn-T activity (7); however, β3Gn-T2, -T3, and -T4 resemble the β3Gal-T family rather than β3Gn-T1. In addition, β3Gn-T1 is structurally distinct from the other members of the β3Gn-T and β3Gal-T families because it does not have 2 of the 3 cysteine residues conserved by all the other members. There was no direct relationship between the subgroups and chromosomal localizations of the genes (Fig. 1*B*). β3Gn-T1, -T2, -T3, and -T4 exhibited no structural similarity to another type of β3Gn-T (named iGnT) that was isolated by expression cloning using the anti-i-antigen antibody, suggesting that the *in vivo* substrate specificity of iGnT might be quite different from that of other β3Gn-Ts.

In this study, we isolated three types of novel β3Gn-T genes, which enabled us to discriminate the respective enzymes at the molecular level. Considering the enzymatic activities *in vitro* and *in vivo* as well as the expression patterns of the β3Gn-Ts, the respective enzymes are likely to play different roles. The poly-*N*-acetyllactosamine or GlcNAcβ1–3Gal structure appears in glycolipids, keratan sulfate proteoglycans, and human milk oligosaccharides, in addition to *N*- and *O*-glycans of glycoproteins. Therefore, the existence of multiple β3Gn-Ts is not strange. It remains to be determined which β3Gn-T makes which types of sugar chains. Poly-*N*-acetyllactosamines are known to be synthesized at various positions by the concerted action of several glycosyltransferases required for the elongation or formation of specific sugar branches preferred by elongation enzymes. For example, poly-*N*-acetyllactosamines are preferentially formed in the specific branch in complex type *N*-glycans, which are formed by β1,6-*N*-acetylglucosaminyltransferase V (102). In addition, core 2 β1,6-*N*-acetylglucosaminyltransferases 1 and 2 and large I β1,6-*N*-acetylglucosaminyltransferases are branching enzymes critical for elongation with poly-*N*-acetyllactosamines (48, 80, 81, 103–105). Discovery of the multiple β3Gn-Ts in addition to other multiple glycosyltransferases involved in the biosynthesis of poly-*N*-acetyllactosamines (*e.g.* β1,4-galactosyltransferases, β3Gal-Ts, β1,6-*N*-acetylglucosaminyltransferase V, core 2 β1,6-*N*-acetylglucosaminyltransferases, and large I β1,6-*N*-acetylglucosaminyltransferases) indicates that regulation of poly-*N*-acetyllactosamine synthesis may be more complex than previously recognized. Definitive determination of the enzymatic activities and expression patterns of the β3Gn-Ts as well as experiments using knockout mice may provide insight into their functions in physiological and pathological processes.

Recently, Amado *et al.* (76) have reported the existence of four additional members of the β3Gal-T family, although their enzymatic activities were not determined. Based on the characteristics of the primary structures and chromosomal localizations, three of them may correspond to β3Gal-T2, -T3, and -T4.

TABLE V

Quantitative analysis of transcripts of β3Gn-T2, -T3, and -T4 in various human tissues and cells by competitive RT-PCR

Cell name	Relative amount of β3Gn-T transcript		
	β3Gn-T2	β3Gn-T3	β3Gn-T4
	β3Gn-T/β-actin × 10²		
Adrenal gland	10	<0.1	<0.1
Whole brain	3.7	<0.1	0.6
Brain amygdala	6.5	<0.1	0.5
Brain caudate nucleus	11	<0.1	0.1
Brain cerebellum	6.4	<0.1	0.2
Brain corpus callosum	2.6	<0.1	<0.1
Brain hippocampus	3.4	<0.1	0.8
Brain substantia nigra	6.3	<0.1	<0.1
Brain thalamus	9.0	<0.1	0.1
Bone marrow	4.6	<0.1	<0.1
Heart	12	<0.1	<0.1
Kidney	9.0	0.3	0.4
Liver	1.4	<0.1	<0.1
Lung	8.1	<0.1	<0.1
Lymph node	6.3	<0.1	<0.1
Mammary gland	10	0.3	<0.1
Pancreas	21	0.9	<0.1
Pituitary gland	20	<0.1	<0.1
Placenta	5.4	0.7	<0.1
Prostate	3.5	<0.1	<0.1
Salivary gland	6.8	0.4	<0.1
Skeletal muscle	1.2	0.3	<0.1
Small intestine	5.1	<0.1	<0.1
Spinal cord	3.3	<0.1	<0.1
Spleen	3.7	<0.1	<0.1
Stomach	4.7	<0.1	<0.1
Testis	3.8	<0.1	<0.1
Thymus	4.2	<0.1	<0.1
Thyroid	2.6	<0.1	<0.1
Trachea	2.8	0.6	<0.1
Uterus	2.4	<0.1	<0.1
Fetal brain	3.8	<0.1	0.1
Fetal kidney	7.7	<0.1	<0.1
Fetal liver	10	<0.1	<0.1
Fetal lung	3.3	<0.1	<0.1
Colon	1.4	7.5	0.4
Esophagus	4.2	1.3	0.3
Jejunum	0.46	2.4	<0.1
Stomach body	1.5	2.3	<0.1
Stomach antrum	1.1	1.5	<0.1
Colo205	23	8.0	<0.1
HL-60	5.3	<0.1	<0.1
Namalwa KJM-1	3.3	<0.1	<0.1

Additional members of the β3Gn-T and β3Gal-T families remain to be investigated.

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Note Added in Proof—Recently, Zhou *et al.* (7) have corrected the nucleotide sequence and deduced amino acid sequence of the β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T1) cDNA that they had previously published (see "Corrections" in *Proc. Natl. Acad. Sci. U.S.A.* (2000) **97**, 11673–11975). This developed from an unfortunate cDNA clone substitution in their laboratory. The corrected sequence of β3Gn-T1 was identical to that of β3Gn-T2. Consequently, besides iGnT, there are three types of β3Gn-Ts described to date, not four. The sequence of β3Gn-T1 used in this paper is that of the corrected, substituted cDNA clone. To try to prevent further confusion, we point out this fact but do not change the enzyme names used in this paper.

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